Protein purification methods

a practical approach

Edited by

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Preface

Protein purification is key to r
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CHAPTER 4

Separation based on structure

S.ROE

1. INTRODUCTION

This chapter aims to provide guidance in the selection of techniques and operating conditions for the purification of proteins based on some of their structural properties. Each technique uses a single parameter (e.g. charge or hydrophobicity) to effect adsorption and separation. In contrast, separation based on biological interaction, which is mostly mediated through a combination of such interactions is covered in the next chapter.

Some general concepts are described in the first instance followed by sections on matrices and equipment which are common to all the techniques. This is followed by description of the theory and practice of individual techniques. The chapter is aimed at those not familiar with these techniques and who will be considering protein purification on a laboratory scale. For further information on this subject the reader is directed to references 1–11.

2. GENERAL CONCEPTS

2.1 Chromatography

Chromatography is the differential separation of sample components between a mobile phase and a stationary phase. In the majority of applications the stationary phase consists of spherical particles which are packed into a column. A mixture of proteins to be separated is introduced into the mobile phase and allowed to migrate through the column. Those proteins having a greater attraction for the solid phase migrate slower than proteins more attracted to the mobile phase, thus effecting resolution (*Figure 1*).

2.2 Adsorption/desorption

While purification by chromatography may provide excellent resolution of protein mixtures adsorption/desorption is of equal importance, particularly for preparative work. This technique also separates proteins according to their relative distribution between a liquid and a solid phase, but is not based on speed of migration. It is generally used in a batch mode to provide a rapid method of concentration and purification, most usefully at an early stage of a process.

2.3 Compatibility

Table 1 shows that each method of purification places certain requirements on the sample which is to be purified. In the design of a purification procedure it is important that

Separation based on structure

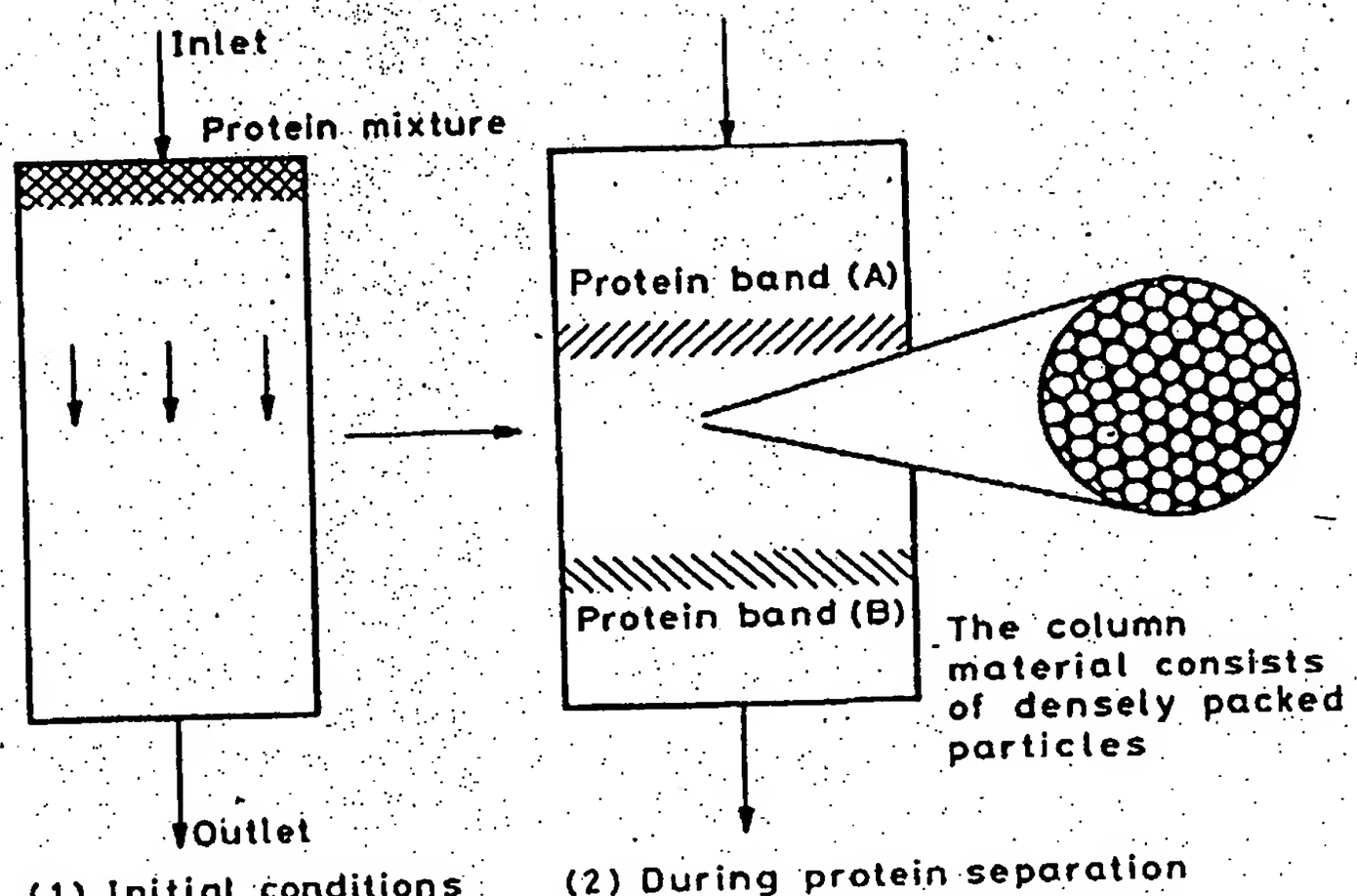


Figure 1. The principle of protein purification using chromatography. A protein mixture is applied to a column in the mobile phase. As it travels down the column proteins are separated depending on their degree of attraction for the column packing. Protein band (A) is more attracted to the stationary phase and moves slower, than protein band (B).

consecutively used techniques are therefore compatible. After a salt precipitation step using ammonium sulphate, for example, the salt concentration of the protein-containing solution is high and therefore unsuitable for the effective use of ion exchange without prior desalting or dilution to reduce the ionic strength. Hydrophobic interaction, however, requires a high salt concentration to promote protein adsorption and may therefore be used after ammonium sulphate precipitation with minimal necessary adjustments to the sample. Elution of proteins in hydrophobic interaction is frequently accomplished using a reduced ionic strength, allowing the subsequent use of an ion exchange step. Thus the selection of technique is clearly dependent on its position in a purification scheme.

2.4 Capacity

Capacity is a measure of the amount of protein which can be adsorbed from solution onto a unit volume or weight of the stationary phase. High capacity techniques can remove proteins from large volumes of solution and are therefore frequently used early on in a purification strategy where both the total protein level and solution volume are high. Ion exchange is an example of a high capacity technique often used to concentrate and purify proteins at an early stage of a purification.

2.5 Selectivity

Selectivity measures the ability of a purification technique to adsorb a protein from

Table 1. Characteristics of the purification

Separation techniques	*Characteristics*	*San Bef*
Ion exchange	High capacity; used in batch or column mode	Lov stre con
Hydrophobic interaction	High capacity; useful after salt precipitation	Hig stre
Hydroxylapatite	Mild separation conditions	Neu low
Metal chelate	Not sensitive to ionic strength	Abs chel
Covalent	Matrix requires lengthy regeneration	Neu mild acidi
Chromatofocusing	High resolution	Low stren

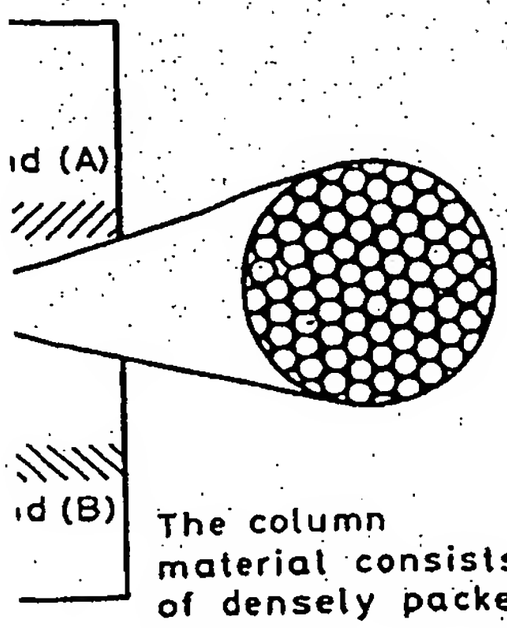
solution with a high degree of reject usually reserved for the last stages in the very similar proteins remaining a selective techniques include affinity

2.6 Resolution

Resolution of proteins is the aim of e of separation of a required protein selectivity can therefore separate Chromatography achieves better resc of separation is improved by multiple front migrates down a column. Ch separation of similar proteins while resolution techniques include chrom fast flow rates and small diameter par

2.7 The efficiency of a separation

The aim of a protein separation is t minimum amount of time. This is call can be improved in two ways.



The column material consists of densely packed particles

protein separation

tography. A protein mixture is applied to a teins are separated depending on their degree : attracted to the stationary phase and moves

atible. After a salt precipitation step ncentration of the protein-containing ffective use of ion exchange without strength. Hydrophobic interaction, romote protein adsorption and may cipitation with minimal necessary ydrophobic interaction is frequently owing the subsequent use of an ion clearly dependent on its position in

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technique to adsorb a protein from

Table 1. Characteristics of the purification techniques discussed in this chapter.

Separation techniques	*Characteristics*	*Sample composition*		*Approx. matrix cost £/litre*	*Stage of purification*
		Before	*After*		
Ion exchange	High capacity; used in batch or column mode	Low ionic strength; correct pH	Change in pH; high ionic strength	200	Variable but especially early
Hydrophobic interaction	High capacity; useful after salt precipitation	High ionic strength	Change in pH and/or low ionic strength	400	Variable but especially early
Hydroxylapatite	Mild separation conditions	Neutral pH; low phosphate	Neutral pH; high phosphate concentration	280	Variable
Metal chelate	Not sensitive to ionic strength	Absence of chelators	Altered pH, presence of chelators	1000	Variable
Covalent	Matrix requires lengthy regeneration	Neutral at mildly acidic pH	Presence of low molecular weight thiols	2000	Late
Chromatofocusing	High resolution	Low ionic strength	Presence of amphoteric buffer. pH close to protein pI.	430	Late

solution with a high degree of rejection of contaminants. High selectivity methods are usually reserved for the last stages in a purification, since they are more able to separate the very similar proteins remaining after earlier general processing. Examples of highly selective techniques include affinity, covalent and metal chelate chromatography.

2.6 Resolution

Resolution of proteins is the aim of each purification step. It is a measure of the degree of separation of a required protein from contaminants. Methods which have a high selectivity can therefore separate proteins with a high degree of resolution. Chromatography achieves better resolution than adsorption/desorption since the degree of separation is improved by multiple opportunities for re-equilibration as the developing front migrates down a column. Chromatography is therefore ideally suited to the separation of similar proteins while adsorption allows protein concentration. High resolution techniques include chromatofocusing and HPLC. The latter technique uses fast flow rates and small diameter particles to provide rapid high resolution separations.

2.7 The efficiency of a separation

The aim of a protein separation is to purify the maximum amount of protein in the minimum amount of time. This is called the throughput. The throughput of a separation can be improved in two ways.

Separation based on structure

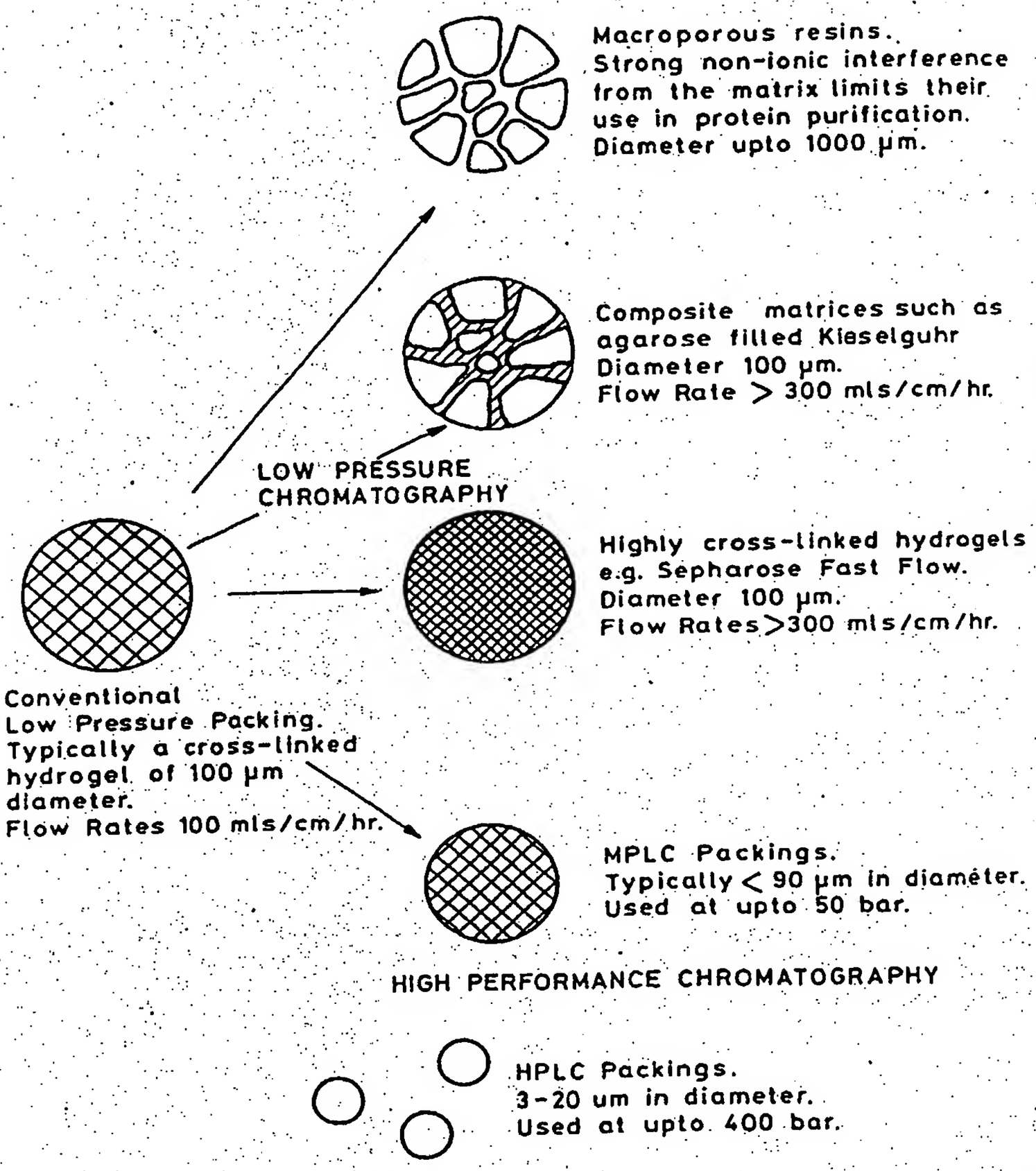

Figure 2. Improvements in matrix design. The variety of stationary phases used in protein purification may be broadly divided between high performance and low pressure matrices.

(i) By the use of high capacity matrices which allow a large amount of protein to be purified at a time.

(ii) By using matrices which can be packed into a column and used at a high flow rate.

Improvements in matrix design have been achieved by using two different methods (*Figure 2*). Firstly, more rigid matrices have been designed by improving the flow

Table 2. Typical characteristics of low and

Characteristic
Particle size (µm)
Flow rate (ml cm^{-2} h^{-1})
Operating pressure (bar)
Separation time (h)
Sample volume
Purification stage
Resolution
Drawbacks

characteristics of existing chromatog
ing a higher degree of matrix cross-l
with a rigid incompressible structure
used without causing bed compressi

Secondly, an alternative approach
been in the design of more rigid mat
performance (pressure) liquid chromat
in part, from the reduction of diffus
resolution achievable in low pressure
reduced since high flow rates (100–3
used in the very evenly packed column
A comparison of the characteristics

Purifications in which the operatin
HPLC (i.e. 6–50 bar) are termed m
These techniques will be discussed i

2.8 Cost

The cost of each matrix and its re-u
Generally those techniques providing
to use (*Table 1*). Consequently high
purification when sample volume is
removed, thereby requiring smaller m
and reagent costs in large-scale use a
this volume *Protein Purification Ap*

3. MATRIX MATERIALS

The matrix is the solid substrate of the
properties to the support by the chem
the same functionality may be availa
to understand the physicochemical pr
influence the selection of a stationar

Macroporous resins.
Strong non-ionic interference from the matrix limits their use in protein purification.
Diameter upto 1000 μm.

Composite matrices such as agarose filled Kieselguhr
Diameter 100 μm.
Flow Rate > 300 mls/cm/hr.

Highly cross-linked hydrogels
e.g. Sepharose Fast Flow.
Diameter 100 μm.
Flow Rates >300 mls/cm/hr.

HPLC Packings.
Typically < 90 μm in diameter.
Used at upto 50 bar.

ICE CHROMATOGRAPHY

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allow a large amount of protein to

column and used at a high flow rate.

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Table 2. Typical characteristics of low and high pressure chromatography.

Characteristic	*Low pressure*	*High pressure*
Particle size (μm)	100	10
Flow rate (ml cm^{-2} h^{-1})	10–30	100–300
Operating pressure (bar)	<5	>50
Separation time (h)	Up to 24	1–3
Sample volume	Variable (ml–litres)	Small (μl–ml)
Purification stage	Variable	Usually late
Resolution	Good	May be excellent
Drawbacks	Long process time; potential need for cold room facilities	High cost of equipment. Risk of denaturation from high pressure

characteristics of existing chromatographic materials. This has been accomplished using a higher degree of matrix cross-linking and by the design of composite materials with a rigid incompressible structure. These materials allow higher flow rates to be used without causing bed compression.

Secondly, an alternative approach to improving both throughput and resolution has been in the design of more rigid matrices of smaller particle size (3–20 μm) in high performance (pressure) liquid chromatography (HPLC). The improved efficiency results, in part, from the reduction of diffusion effects which are responsible for the lower resolution achievable in low pressure liquid chromatography (LPLC). Diffusion is also reduced since high flow rates (100–300 ml cm^{-2} h^{-1}) at high pressure (>50 bar) are used in the very evenly packed columns usually supplied ready packed by manufacturers. A comparison of the characteristics of LPLC and HPLC is given in *Table 2*.

Purifications in which the operating pressure lies between those used in LPLC or HPLC (i.e. 6–50 bar) are termed medium pressure liquid chromatography (MPLC). These techniques will be discussed in detail in subsequent sections.

2.8 Cost

The cost of each matrix and its re-usability are important considerations in its use. Generally those techniques providing a greater degree of selectivity are more expensive to use (*Table 1*). Consequently high selectivity techniques tend to be used later in the purification when sample volume is low and many contaminants have already been removed, thereby requiring smaller matrix volumes. Implications of matrix, equipment and reagent costs in large-scale use are discussed in more detail in the companion to this volume *Protein Purification Applications: A Practical Approach* (see ref. 12).

3. MATRIX MATERIALS

The matrix is the solid substrate of the stationary phase. It is modified to confer specific properties to the support by the chemical attachment of various functional groups. As the same functionality may be available on a wide range of matrices it is important to understand the physicochemical properties of the matrices and consider how these influence the selection of a stationary phase (*Table 3*).

Table 3. Some characteristics of commonly used matrices.

Matrix	*Manufacturers*	*pH stability*	*Typical cross-linker*	*Drawbacks of use*
Cellulose	Whatman Pharmacia-LKB Bio-Rad, Serva	3–10 2–12 if cross-linked	Epichlorohydrin	Cycling is required for dry material
Dextran	Pharmacia-LKB	2–12	Epichlorohydrin	Swells and shrinks depending on ionic strength
Agarose	Bio-Rad Pharmacia-LKB	4–9 3–14 if cross-linked	2,3-dibromopropanol	Must not dry out
Polyacrylamide	IBF Bio-Rad Pharmacia-LKB	2–11	*N-N'*-Methylene bisacrylamide	*N-N'* bis is toxic
Silica	Whatman Waters DuPont Merck	3–8	By poly-condensation	Unstable above pH8
Polystyrene	Rohm & Haas Dowex Bio-Rad	Any pH	Divinyl benzene	Non-ionic interaction

Important criteria in the choice of a matrix are as follows.

(i) High mechanical stability: allows maximization of flow rate, minimizes the pressure drop and results in low abrasion.

(ii) Good chemical stability: necessary to maintain the bed structure, allow sterilization and minimize contamination of the protein mixture.

(iii) High capacity: for minimizing the bed volume thus maximizing speed of operation. The capacity is dependent on the density of groups available for functionalization.

(iv) In porous matrices pore size must be sufficiently large to allow access of proteins to the internal surface.

(v) Pore shape is also important since blocking of pore entrances can dramatically reduce available surface areas.

(vi) The surface of the matrix should be inert in order to minimize non-specific adsorption which can eventually result in fouling of the matrix.

(vii) Matrix density should be suitable for the application; e.g. fluidized beds require densities to be sufficiently high so as to maintain a stable bed.

(viii) Particle size influences the choice of equipment required to carry out a separation. The mass transfer of solutes from the mobile phase onto the stationary phase is largely determined by the diffusion distance (6,7). If smaller particles are used specialized chromatographic equipment must be available to deliver an even flow of solvent at high back pressures.

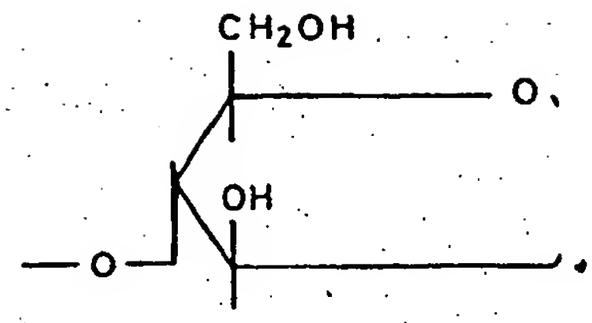

Figure 3. The chemical structure of cellu

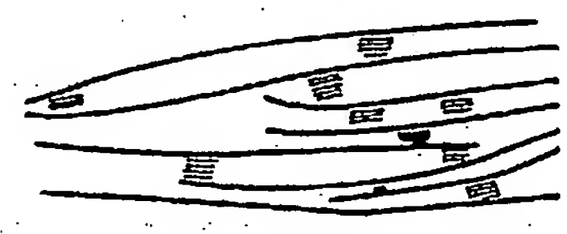

Figure 4. The result of cycling dry cellulose and widening of the matrix pores (b).

3.1 Cellulose

This is a linear polysaccharide of glucose residue has three hydroxy and easily derivatized. Cellulose it towards mineral acids, alkalis and o pH range of 3–10 but can withstan are formed at regions of the polyme is reduced. The nature of these por of cellulose. It is necessary to pre-tu to swell the matrix and open up t

Cellulose is available in fibrous, has good hydrodynamic propertie in early purification steps. Microgra rigidity and porosity are increased b Both matrices are unstable at high a further improvement in rigidity a properties.

3.2 Agarose

Agarose is a polysaccharide obtain is composed of polymeric chains 3,6-anhydro-1-galactose) (*Figure* ture upon cooling hot solutions con (*Figure 6*) (13).

The matrix is highly porous wi

Typical cross-linker	*Drawbacks of use*
Epichlorohydrin	Cycling is required for dry material
Epichlorohydrin	Swells and shrinks depending on ionic strength
1,3-dibromopropanol	Must not dry out
N-N'-Methylene bisacrylamide	*N-N'* bis is toxic
By poly-condensation	Unstable above pH8
Divinyl benzene	Non-ionic interaction

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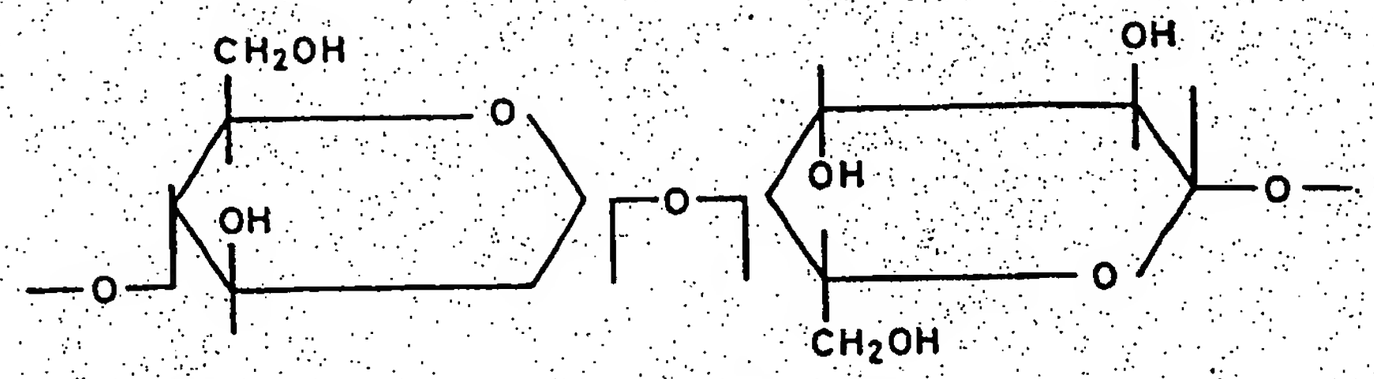


Figure 3. The chemical structure of cellulose: $\beta 1-4$ linked glucose residues.

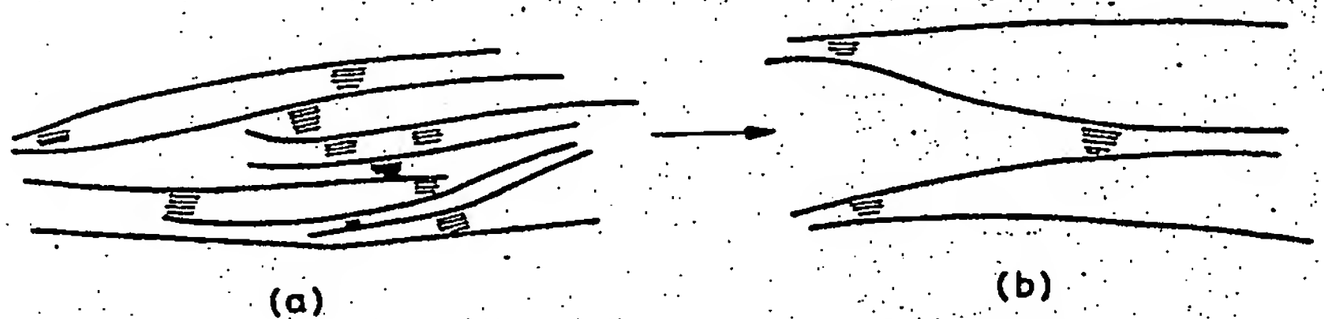


Figure 4. The result of cycling dry cellulose (a) in 0.5 M NaOH leads to an opening up of the fibrous structure and widening of the matrix pores (b).

3.1 Cellulose

This is a linear polysaccharide of $\beta 1-4$ linked glucose monomers (*Figure 3*). Each glucose residue has three hydroxyl groups which makes the matrix very hydrophilic and easily derivatized. Cellulose itself is relatively inert towards proteins and unstable towards mineral acids, alkalis and oxidants. Cellulosic ion exchangers have an operating pH range of 3–10 but can withstand 0.5 M alkali for up to 2 h. The pores in cellulose are formed at regions of the polymer structure where hydrogen bonding between chains is reduced. The nature of these pores depends on the degree of solvation and the batch of cellulose. It is necessary to pre-treat dry cellulose in 0.5 M alkali for 30 min in order to swell the matrix and open up the pores (*Figure 4*).

Cellulose is available in fibrous, microgranular or bead form. The fibrous matrix has good hydrodynamic properties (400 ml cm^{-2} h^{-1}) and is recommended for use in early purification steps. Microgranular cellulose provides better resolution since matrix rigidity and porosity are increased by chemical cross-linking and partial acid hydrolysis. Both matrices are unstable at high flow rates and may collapse. Beaded cellulose is a further improvement in rigidity and chemical stability giving the best hydrodynamic properties.

3.2 Agarose

Agarose is a polysaccharide obtained by purifying agar, a component of seaweed. It is composed of polymeric chains of the disaccharide agarobiose (D-galactose and 3,6-anhydro-1-galactose) (*Figure 5*), and forms a very porous hydrophilic gel structure upon cooling hot solutions containing agarose at concentrations as low as 2% w/v (*Figure 6*) (13).

The matrix is highly porous with minimal non-specific adsorption which can be

Separation based on structure

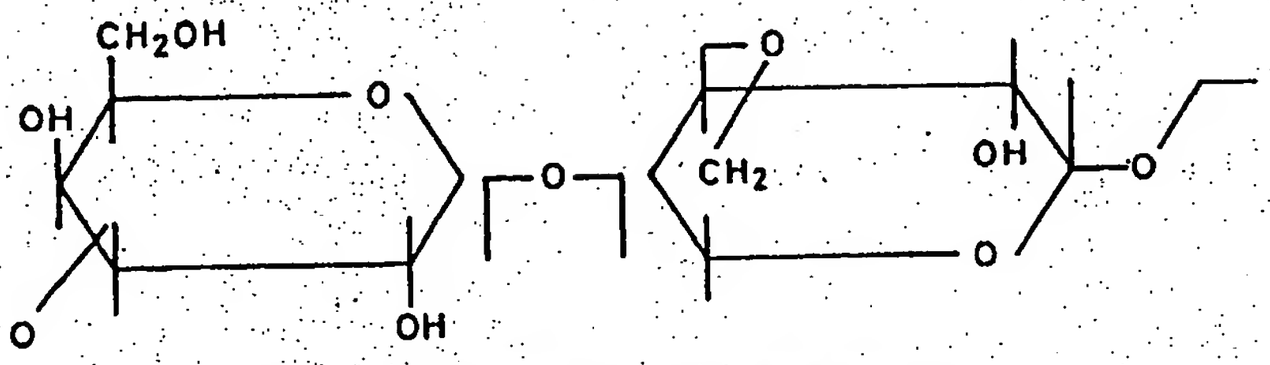


Figure 5. The chemical structure of agarose: the disaccharide repeating unit.

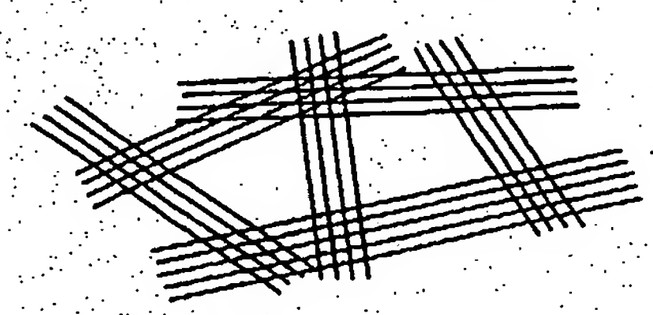


Figure 6. The macrostructure of agarose: a highly porous hydrophilic matrix.

reduced even further in the presence of low concentrations of salt (20 mM). While simple agarose gels have limited stability at extremes of pH, this can be extended by cross-linking with dibromopropanol to provide a gel which can be autoclaved at 120°C and is stable over a wider pH range (3−14). The level of cross-linking and amount of agarose used control the pore size of the gel. Agarose matrices should never be allowed to dry out since they undergo irreversible changes in structure once the structural water is lost.

Cross-linked agarose also has good hydrodynamic properties; flow rates of 10−30 ml cm^{-2} h^{-1} are common. However, a new generation of more highly cross-linked agaroses are now available (e.g. Sepharose Fast Flow) with flow rates up to 300 ml cm^{-2} h^{-1}. In practice, optimum resolution is obtained at 30−60 ml cm^{-2} h^{-1}.

3.3 Dextran

Dextran (4) is an extracellular polysaccharide produced by the bacterium *Leuconostoc mesenteroides* and consists of glucose residues linked by $\alpha 1-6$ bonds (*Figure 7*). Each residue has six hydroxyl groups, providing a very hydrophilic matrix which is relatively chemically inert and easily derivatized. Dextran matrices are less stable than cellulose to acid hydrolysis but can withstand 0.1 M HCl for up to 2 h. The matrix is stable over a wide pH range (2−12) and is cross-linked with epichlorohydrin (*Figure 8*) for use as a gel matrix in column chromatography. As with agarose, the amount of cross-linker used controls the pore size of the matrix. The gels are rather soft, easily compressed and swell considerably in aqueous solutions.

The exclusion limit of commercially used dextran matrices is, at 300 kd (for a globular protein), rather less than agarose. Unlike cellulose, the pore size is relatively homogenous and does not require any pre-treatment other than soaking the matrix to allow swelling. Cross-linked dextrans are autoclavable but biodegradable. Dextrans can be repeatedly dried and swollen without alteration of chromatographic properties. Dextran-based ion exchangers suffer from a strong dependence of bed volume on pH and ionic strength.

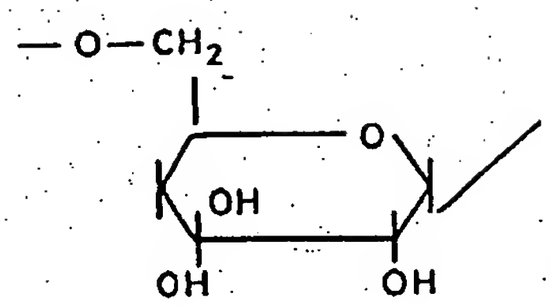
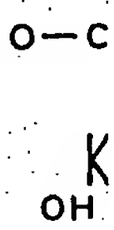


Figure 7. The chemical structure of dextr



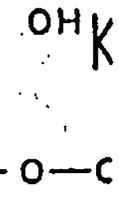


Figure 8. Epichlorhydrin cross-linked dex

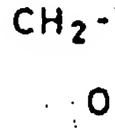
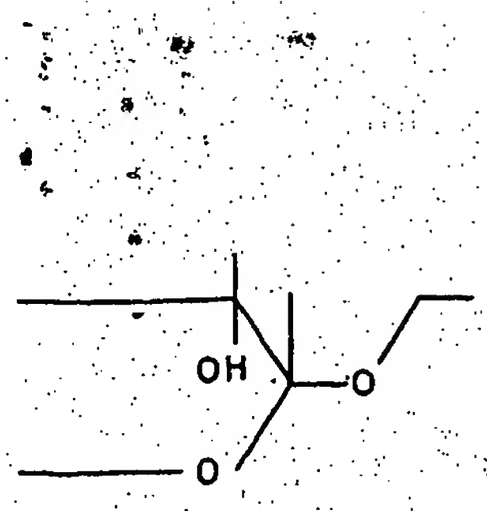


Figure 9. The repeating unit of polyacryl

3.4 Polyacrylamide

Polyacrylamide gels (3) are produce of a cross-linking agent, *N,N'*-meth is carried out in the presence of a cat



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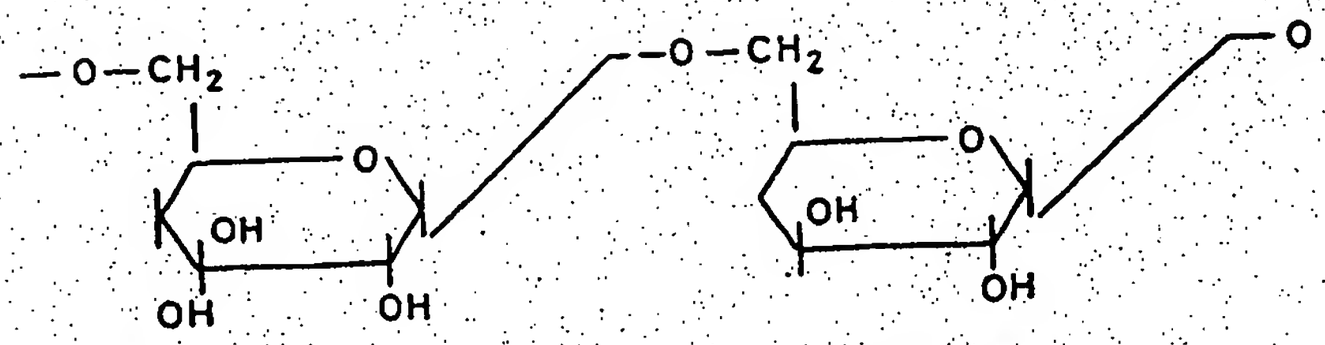

Figure 7. The chemical structure of dextran: α1–6 linked glucose residues.

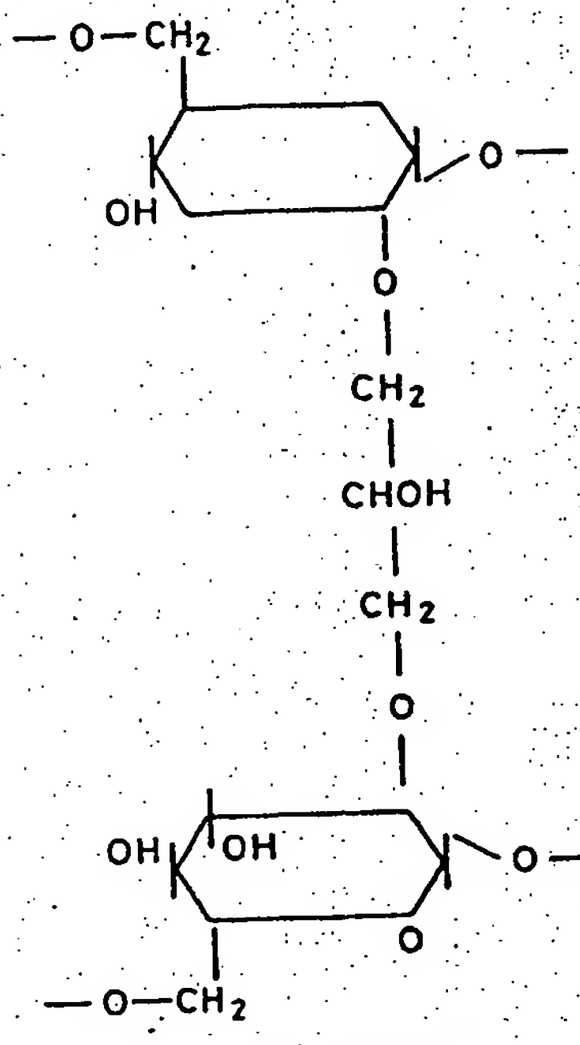

Figure 8. Epichlorhydrin cross-linked dextran (e.g. Sephadex).

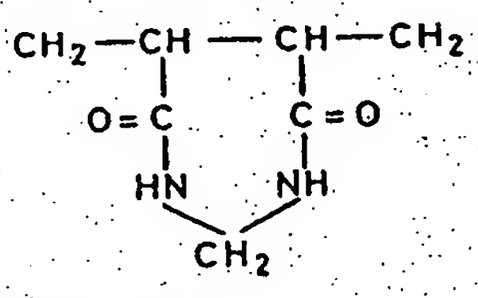

Figure 9. The repeating unit of polyacrylamide.

3.4 Polyacrylamide

Polyacrylamide gels (3) are produced by the polymerization of acrylamide in the presence of a cross-linking agent, *N*,*N*′-methylene bisacrylamide (*Figure 9*). The polymerization is carried out in the presence of a catalyst (e.g. persulphate) and in the absence of oxygen.

Separation based on structure

The hydrophilic compressible gels produced are used in gel permeation and modified to form ion exchangers. Gels are autoclavable and, although chemically stable, toxic acrylamides may slowly leach and may therefore limit their use in food and pharmaceutical applications. Manufacturers of these materials include IBF and Bio-Rad.

3.5 Natural earths

This group of matrices includes Kieselguhr, Fullers Earth, Bentonite and Alumina. These materials may serve without derivatization for the adsorption of proteins although the exact mechanisms involved are not well understood. Kieselguhr, derived from the silica shells of diatoms, has been used most as a matrix for both HPLC and LPLC. In the latter example, inorganic particles are fabricated into macroporous particulate matrices of size 250–500 μm with very large pores (up to 7 μm) and highly stable characteristics. These granules may serve as adsorbents (e.g. Titania) or provide an inert matrix for subsequent use in combination with hydrogels as composite ion exchangers (14). Matrices are incompressible, allowing high flow rates with minimal back pressure in packed beds.

Alumina is formed by the precipitation of sodium aluminate to form aluminium hydroxide which, through thermal dehydration and ageing yields porous alumina aggregates (3). Matrices may be slightly acidic, neutral or basic in water depending on the treatment. Alumina is used in HPLC as porous or pellicular particles (Merck-Lichrosorb, Whatman-Pellumina) or as a batch adsorbent (Bio-Rad, BDH, Sigma).

3.6 Silica

Silica matrices (15), commonly used in HPLC, are formed by the acidification of sodium silicate to form a sol of orthosilicic acid which is subject to polycondensation during ageing to yield silica particles (*Figure 10*). The silanol (Si-OH) groups present at the silica surface make them very hydrophilic and easily derivatized. Silica matrices are incompressible and thus well suited to HPLC. They are unaffected by organic solvents and mechanically stable but gradually dissolve above pH 8. In derivatized silica, unreacted silanol groups provide the matrix with a weak cation exchange nature which is usually masked by end-capping with, for example, trimethylchlorosilane. Silicas are used in HPLC as porous particles or as coatings on glass beads in pellicular packings. Serva also produce silica matrices for low pressure chromatography (Daltosil) using a particle size of up to 200 μm and pore sizes up to 30 nm.

3.7 Porous glass

Controlled pore glass (CPG) is formed from the chemical treatment of alkaline borosilicate glass (16). CPG in bead form has been used in HPLC as a chemically inert, thermally stable matrix. Like silica, it is a negatively-charged aerogel which dissolves in alkaline conditions. CPG has a narrow pore size distribution and although used in HPLC, is a less common matrix than silica. Manufacturers include Serva, BDH and Sigma.

3.8 Polystyrene and phenol-formaldehyde

These organic synthetic polymers (*Figure 11*) are commercially available as 1–2 mm

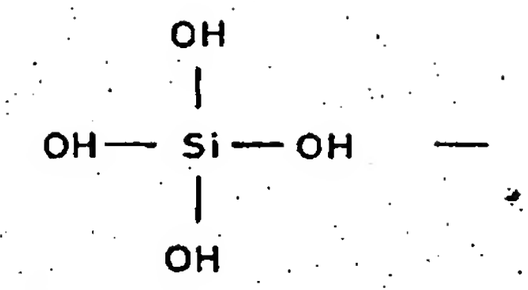

Figure 10. The polycondensation of silicic . remain, giving silica gel its desiccant prope

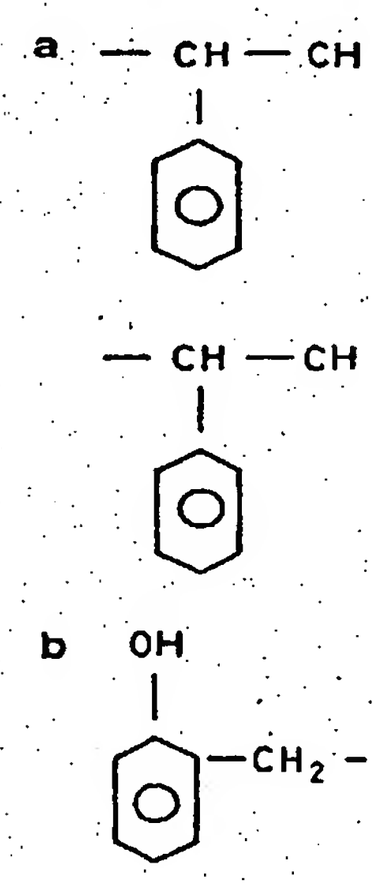

Figure 11. (a) The chemical structure of polyst

beads, called resins. The pore size linking which in turn is determined manufacturing process (4). Although non-ionic in nature, the hydrophilic n ionic groups so that they can be use

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ly derivatized. Silica matrices are
are unaffected by organic solvents
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trimethylchlorosilane. Silicas are
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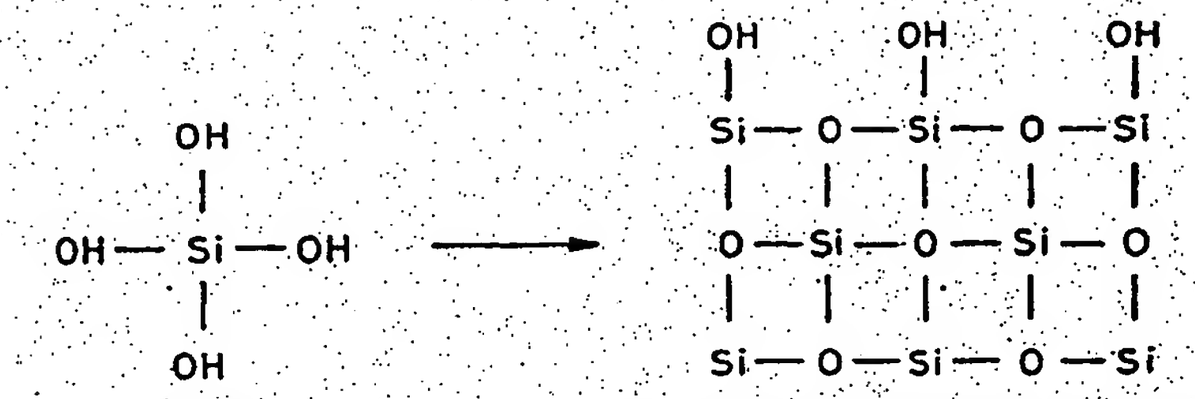


Figure 10. The polycondensation of silicic acid to produce silica gel. At the silica surface, silanol groups remain, giving silica gel its desiccant properties.

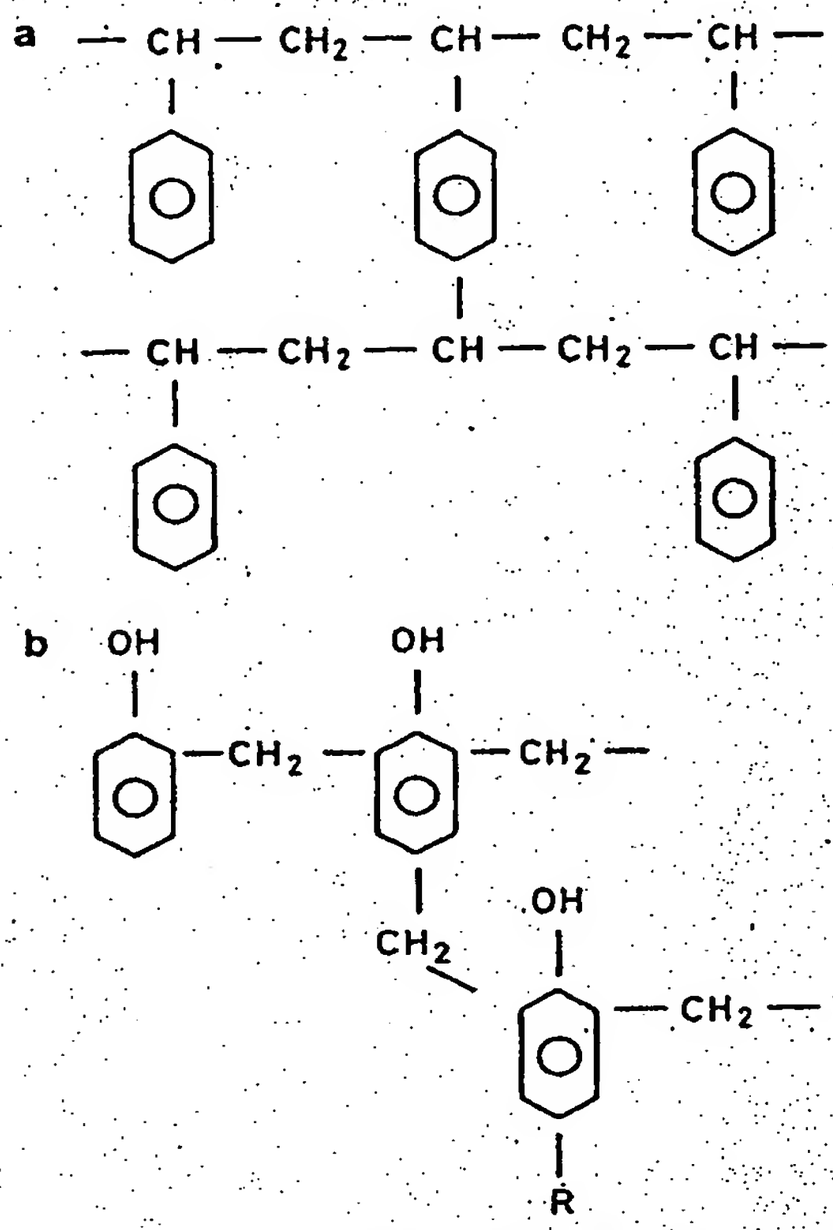


Figure 11. (a) The chemical structure of polystyrene resins (b) The chemical structure of phenol-formaldehyde.

beads, called resins. The pore size of the resins is controlled by the level of cross-linking which in turn is determined by the amount of divinyl benzene used in the manufacturing process (4). Although originally used in water treatment and essentially non-ionic in nature, the hydrophilic nature of the resins has been increased by attaching ionic groups so that they can be used in protein recovery and immobilization (17).

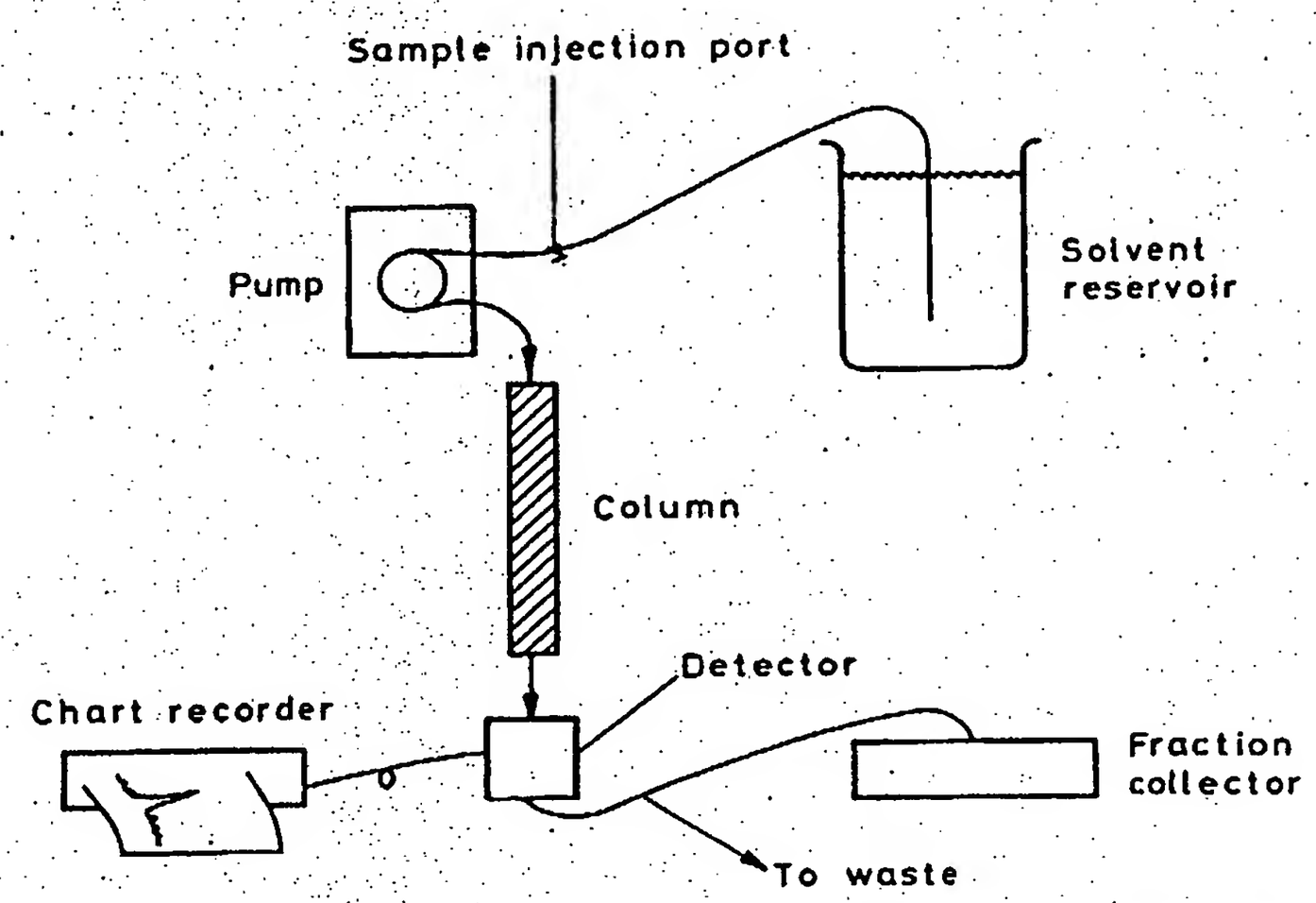


Figure 12. Typical components of low pressure chromatography. Notice that the tube length between the column outlet and the detector is kept small so as to minimize loss of resolution.

4. CHROMATOGRAPHIC EQUIPMENT AND BASIC PROCEDURES

Although proteins are separated using a variety of matrices the basic components of a chromatographic system are similar and consist of the following.

(i) A pump to give an even flow of liquid.
(ii) A column in which the protein separation occurs.
(iii) A detector to provide a continual measurement of a physical parameter of the eluent.
(iv) A recorder to give a continuous visual read-out of the detector output.
(v) A fraction collector to separate the column eluent into samples.

These are typically set up as shown in *Figure 12*. The requirements of each unit, in particular the pump and column, differ depending on the operating pressure. Consequently the equipment and procedures for protein separation will be subdivided as follows:

LPLC: <5 bar
MPLC: 6–50 bar
HPLC: >50 bar

Pressure units can be interconverted using the formula:

1 bar = 15 p.s.i. = 0.1 MPa

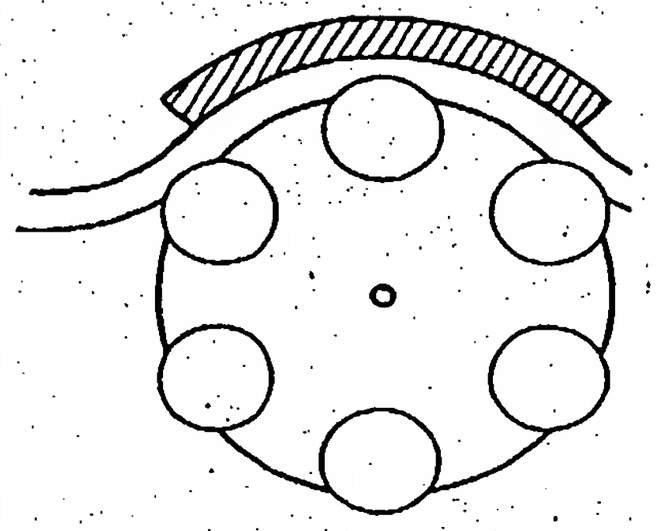


Figure 13. Roller (left) and planetary (right

Table 4. Some commonly used peristaltic

Manufacturer	*Pump*
Bio-Rad	Econ
Pharmacia-LKB	P1
	P3
	P500
Watson-Marlow	–
Whatman	Perist

4.1 Low pressure equipment

4.1.1 *Pumps*

A variable speed pump capable of c
is commonly a peristaltic pump wh
may be of two designs: (i) roller;

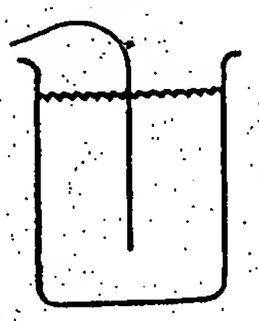
A roller pump directs solvent by
a revolving disc, the amount of co
against a tube guiding plate. A dra
gradually wear.

A planetary pump also directs sc
rub against each other and gradual

Examples of the most commonly
in *Table 4*. Many pumps can indu
simultaneously. Pumps must be ca

4.1.2 *Columns*

Low pressure chromatography colu
facturers in different lengths, sizes
strength (*Table 5*). For simple smal
may be made in the laboratory and
for some separations. A glass colun



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BASIC PROCEDURES

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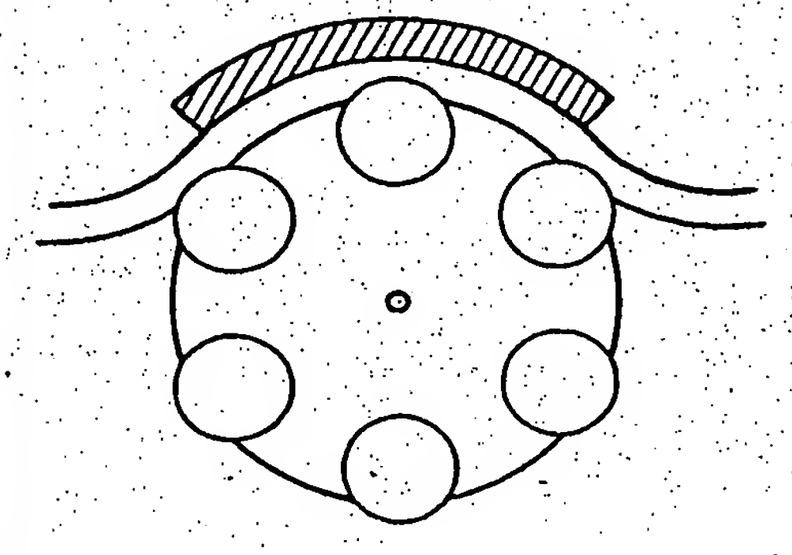

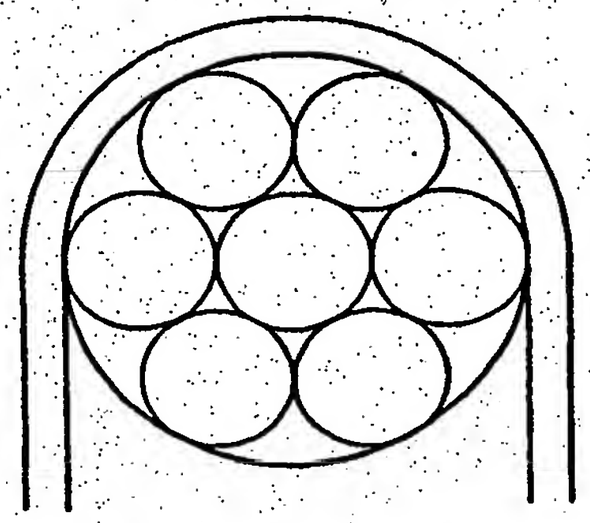

Figure 13. Roller (left) and planetary (right) pump designs commonly used in low pressure chromatography.

Table 4. Some commonly used peristaltic pumps.

Manufacturer	*Pump name*	*Description*
Bio-Rad	Econo-column	2 channel 0.6–325 ml h^{-1}
Pharmacia-LKB	P1	1 channel 0.6–500 ml h^{-1}
	P3	3 channel 0.6–400 ml h^{-1}
	P500	Dual piston, high precision
Watson-Marlow	–	Wide variety available
Whatman	Peristaltic	4 channel 0.03–2280 ml h^{-1}

4.1 Low pressure equipment

4.1.1 *Pumps*

A variable speed pump capable of directing solvent at a low flow rate is suitable. This is commonly a peristaltic pump which uses silicon, PVC or fluoro-rubber tubing and may be of two designs: (i) roller; (ii) planetary as shown in *Figure 13*.

A roller pump directs solvent by tube compression using rollers at the periphery of a revolving disc, the amount of compression being controlled by tightening a screw against a tube guiding plate. A drawback of this type of pump is that the tubing will gradually wear.

A planetary pump also directs solvent by tube compression using rollers but these rub against each other and gradually wear.

Examples of the most commonly available pumps and their specifications are given in *Table 4*. Many pumps can induce flow along several tubes of varying diameters simultaneously. Pumps must be calibrated for a specific diameter of tubing.

4.1.2 *Columns*

Low pressure chromatography columns are available from a large number of manufacturers in different lengths, sizes, degrees of chemical resistance and mechanical strength (*Table 5*). For simple small-scale laboratory experiments, however, columns may be made in the laboratory and, although less exact in their design, are suitable for some separations. A glass column with a fritted glass filter at its base can be used

Table 5. List of some small scale chromatography columns.

Manufacturer or supplier	*Trade name*	*Description*
Alltech	Cheminert	
	MB	Glass, fixed bed, 15–100 cm length
	LC	Glass, adjustable, 33–109 cm length
Amicon	Lab Columns	Glass + polyamide, polypropylene 4–1520 ml volume
Beckman	–	For low–medium pressure Glass and fluorocarbon Adjustable, solvent resistant
Bio-Rad	Econo-columns	Glass, adjustable Up to 590 ml volume
	Bio-Rex MP	Solvent resistant Pressure resistant up to 500 p.s.i. 15–2000 ml volume
Pharmacia-LKB	C-Series	Glass and polypropylene, polyamide, polyethylene and fluoro-rubber Up to 520 ml volume Adjustable, autoclavable
	K-Series	For labile biopolymers Available at different levels of solvent resistance Up to 1800 ml volume Adjustable
	SR Series	Minimal heavy metal contamination
Pierce	Chromato Flo	Glass and polypropylene or teflon Adjustable 19–1176 ml volume
Whatman	IEC	Glass and polypropylene volume up to ~500 ml

for separations provided a tube can be fitted to the column top to supply a solvent inlet. The glass frit must retain the adsorbent particles but must not restrict flow.

Commercially available columns used in small-scale separations are usually made of glass with a variety of internal diameters and column lengths to give a packed bed volume of up to 2 litres. The volume of most columns may be adjusted using adaptors mounted at the inlet and/or outlet. All these columns are of modular design so that their end fittings can be interchanged for cleaning. Many columns are jacketed for temperature control and are autoclavable. Where columns cannot be autoclaved, sterilization with ethylene oxide is recommended. The sorbent bed is held in place by a mesh (usually 20 μm) while the inlet may be fitted with a reversed funnel design to provide an even solvent distribution over the packed bed.

4.1.3 *Detectors*

Detectors may measure UV absor
optical density. The strong absorpt
is due to peptide bonds and aron
therefore the most common methoc
detectors may provide improved se
to detection means that this meth

Ultraviolet detectors used in low
or zinc lamps. The UV light from t
and then through an optically clear
intensity due to absorption by the
This generates a signal (0–10 mV
of the detector can be adjusted to

The Pharmacia UV1 detector m
mercury lamp while the UV2 has tv
pathlengths (20 mm and 1 mm) o
sensitivity from the same detector

Bio-Rad market two detectors. TI
tor which can be used on any scale
The 1740 model monitors at 254 ar

4.1.4 *Fraction collectors*

Fraction collectors allow the auto
after they pass through the detect
volume of each sample can be
programmable, allowing the colle
of washing and elution cycles.

4.2 Basic procedures in low pre

The basic procedures in establishi

4.2.1 *Choice of column dimensio*

The optimum size and dimension
factors which include the sample s
ty and the type of matrix used.

Traditionally chromatography cc
leads to a low flow rate and long s
required in order to minimize gel
Both column designs have their
maximizing peak resolution, the l
protein activity in some applicatic
control with wide bore columns so t
to loss of resolution. Hydrogel mat

Description
Glass, fixed bed, 15–100 cm length Glass, adjustable, 33–109 cm length
Glass + polyamide, polypropylene 4–1520 ml volume
For low–medium pressure Glass and fluorocarbon Adjustable, solvent resistant
Glass, adjustable Up to 590 ml volume Solvent resistant Pressure resistant up to 500 p.s.i. 15–2000 ml volume
Glass and polypropylene, polyamide, polyethylene and fluoro-rubber Up to 520 ml volume Adjustable, autoclavable For labile biopolymers
Available at different levels of solvent resistance Up to 1800 ml volume Adjustable Minimal heavy metal contamination
Glass and polypropylene or teflon Adjustable 19–1176 ml volume
Glass and polypropylene volume up to –500 ml

column top to supply a solvent inlet.
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ımns are of modular design so that
ıg. Many columns are jacketed for
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The sorbent bed is held in place by
ted with a reversed funnel design to
:ked bed.

4.1.3 *Detectors*

Detectors may measure UV absorbance, fluorescence, conductivity, radioactivity or optical density. The strong absorption of light by proteins at 206–215 nm and 280 nm is due to peptide bonds and aromatic amino acids, respectively. UV absorption is therefore the most common method of protein detection used and although fluorescence detectors may provide improved sensitivity, the necessity for sample derivatization prior to detection means that this method of detection is less common.

Ultraviolet detectors used in low pressure chromatography commonly have mercury or zinc lamps. The UV light from the lamp passes through the stream of an eluent liquid and then through an optically clear silica continuous flow cell. The diminution in light intensity due to absorption by the proteins is detected by a highly sensitive photocell. This generates a signal (0–10 mV) which is passed to a chart recorder. The sensitivity of the detector can be adjusted to alter the output signal.

The Pharmacia UV1 detector monitors at 254, 280 or 405 nm using a low pressure mercury lamp while the UV2 has two independent monitoring systems using two optical pathlengths (20 mm and 1 mm) on the same flow cell. This provides two levels of sensitivity from the same detector allowing the measurement of low levels of protein.

Bio-Rad market two detectors. The 1306 is a variable wavelength low noise UV detector which can be used on any scale of chromatography from microbore to preparative. The 1740 model monitors at 254 and 280 nm by changing filters using a mercury lamp.

4.1.4 *Fraction collectors*

Fraction collectors allow the automatic collection of samples taken from the column after they pass through the detector. The number of fractions taken and the time or volume of each sample can be pre-set. Many fraction collectors (*Table 6*) are programmable, allowing the collection of only the desired protein peak or the control of washing and elution cycles.

4.2 Basic procedures in low pressure chromatography

The basic procedures in establishing any LPLC purification step are discussed below.

4.2.1 *Choice of column dimensions*

The optimum size and dimension of a chromatography column will depend on several factors which include the sample size, the protein concentration, the adsorbent capacity and the type of matrix used.

Traditionally chromatography columns are long and thin (i.e. high aspect ratio). This leads to a low flow rate and long separation times. However, short, wide columns are required in order to minimize gel compression; flow rates are consequently higher. Both column designs have their drawbacks. Long, thin columns are excellent for maximizing peak resolution, the longer process time may, however, lead to loss of protein activity in some applications (8). Even a well distributed flow is less easy to control with wide bore columns so that channelling of solvent flow is more likely, leading to loss of resolution. Hydrogel matrices are commonly used in stacked column arrange-

Table 6. List of some commonly used fraction collectors.

Manufacturer	*Name*	*Details*
LKB	UltroRac	200 tubes maximum Three collection modes (time, volume, drop)
	SuperRac	312 tubes maximum. Time, volume, drop modes. Programmable
	HeliRac RediRac	Compact design, external control, 160 tubes maximum. Time, volume or drop mode
Pharmacia	Frac 100	Up to 95 tubes. Automated or manual
	Frac 200 Frac 300	Up to 300 tubes. Microprocessor controlled

Table 7. Sample loading requirements for different methods of protein purification.

Purification method	*% of bed volume required for loading*
Adsorption (concentration)	Up to 100
Stepwise gradient	Up to 50
Continuous gradient	5–10
Isocratic	1–5

ments in large-scale purification. More rigid matrices, typified by the new series of Macrosorb type adsorbents, can be used at a high flow rate in long, narrow columns without pressure build-up and matrix compression. The choice of optimum dimension is therefore clearly dependent on matrix type; this becomes more critical in large-scale process chromatography where back pressures are more significant.

The total volume of matrix required will depend on the capacity of the adsorbent and the protein content of the sample. A method for calculating adsorbent capacity is given in Section 4.2.2. Manufacturers' data on adsorbent protein capacity should always be treated as an upper limit since they are calculated under conditions of excess protein. Working capacities are frequently much less and dependent on the protein size and sample characteristics (pH, ionic strength, presence of contaminants). Working capacities of ion exchangers are usually around 30 mg ml^{-1} of matrix when using complex protein mixtures (8).

For stepwise elution the sample should be loaded onto approximately half the packed bed volume leaving the other half to effect separation during elution. The bed volume for elution is kept small to minimize band spreading through diffusion during stepwise elution. Columns are consequently short. In gradient elution, however, longer columns are required and best resolution is usually obtained if the top 10% of the bed is used for initial sample loading. A longer column can be used since band spreading is minimized due to the continuously changing eluting conditions.

In some purifications the primary objective of the step is concentration rather than

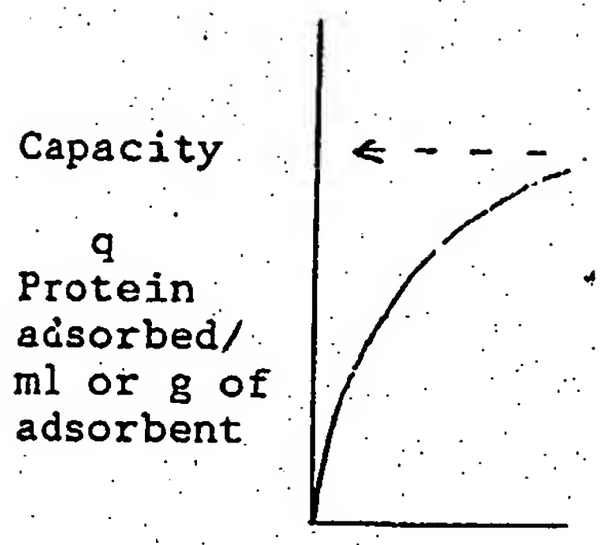

Figure 14. Adsorption isotherm used for

fractionation. In this technique the is common. The total bed capaci allowing the concentration of prot bed. The required protein is then

The calculation of the required purification: these are summarize

4.2.2 *Calculation of adsorbent ca*

The capacity of an adsorbent is d protein, the adsorption conditions a matrices should be used for high adsorption of very large protein preferable. A matrix should theref for the protein to be purified.

The capacity should always be c In particular the pH, ionic strength approximate indication of an adsor onto a small packed bed under the ac in the eluent (i.e. breakthrough). Al for the protein may be determine adsorption of protein from a fixed vo The adsorption can be carried out i gently mixed until equilibrium is rea at equilibrium is then measured. F weight of adsorbent (q) is calculate be drawn by plotting q against c (*Fi* in mg of protein adsorbed per unit maximum volume or concentration of adsorbent can then be determined. then depends on the method of pu

Details
00 tubes maximum hree collection modes (time, volume, drop)
12 tubes maximum. Time, volume, drop odes. Programmable
ompact design, external control, 50 tubes maximum. Time, volume or drop ode
p to 95 tubes. Automated or manual
p to 300 tubes. Microprocessor controlled

of protein purification.

% of bed volume required for loading
Up to 100
Up to 50
5–10
1–5

rices, typified by the new series of
flow rate in long, narrow columns
. The choice of optimum dimension
ecomes more critical in large-scale
e more significant.
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rbent protein capacity should always
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and dependent on the protein size
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30 mg ml^{-1} of matrix when using

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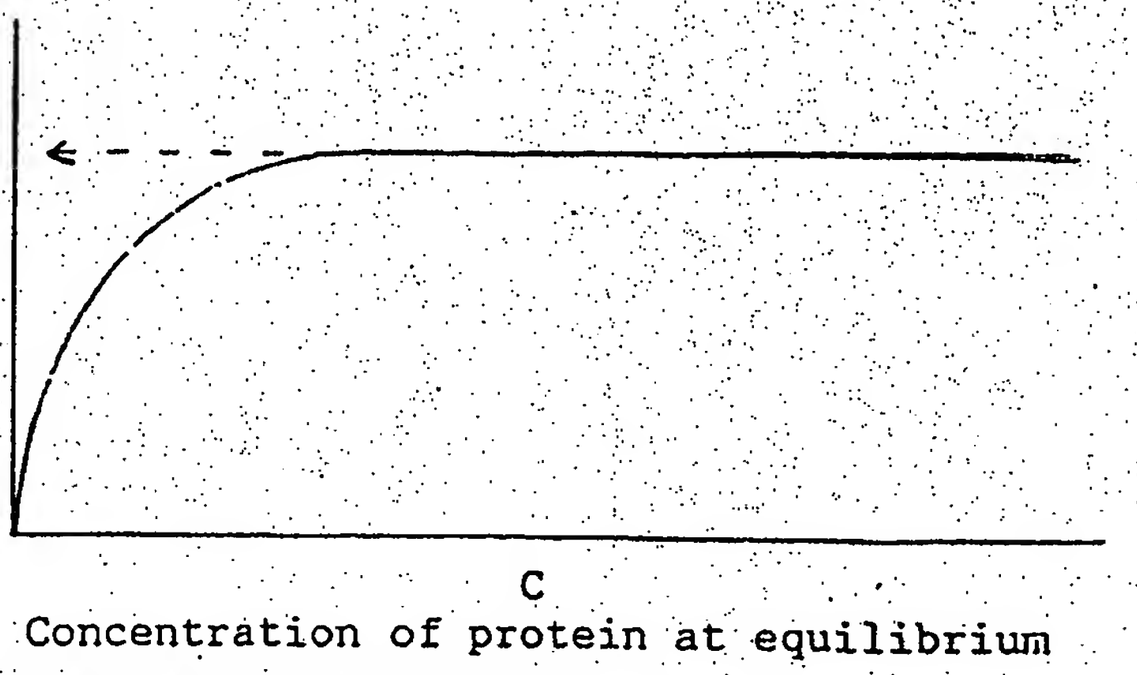

Figure 14. Adsorption isotherm used for the calculation of adsorbent capacity.

fractionation. In this technique the process of adsorption rather than chromatography is common. The total bed capacity may therefore be used for the adsorption step, allowing the concentration of protein from a large volume of sample onto a packed bed. The required protein is then eluted in a small volume.

The calculation of the required bed volume therefore depends on the method of purification: these are summarized in *Table 7*.

4.2.2 *Calculation of adsorbent capacity*

The capacity of an adsorbent is dependent on the molecular weight of the required protein, the adsorption conditions and the nature of the sample. Generally more porous matrices should be used for high molecular weight proteins (>100 kd). For the adsorption of very large proteins ($>4 \times 10^3$ kd) surface adsorption is usually preferable. A matrix should therefore be selected with an adequate degree of porosity for the protein to be purified.

The capacity should always be calculated using the conditions used for adsorption. In particular the pH, ionic strength and level of contaminants should be identical. An approximate indication of an adsorbent capacity can be obtained by loading a sample onto a small packed bed under the adsorption conditions until the desired protein appears in the eluent (i.e. breakthrough). Alternatively, the adsorption isotherm of the adsorbent for the protein may be determined. This can be calculated by measuring the batch adsorption of protein from a fixed volume of sample onto variable volumes of adsorbent. The adsorption can be carried out in test tubes in which the adsorbent and sample are gently mixed until equilibrium is reached. The amount of the protein left in each solution at equilibrium is then measured. From this the amount adsorbed per unit volume or weight of adsorbent (q) is calculated. The adsorption isotherm for the protein can then be drawn by plotting q against c (*Figure 14*) from which the capacity Q_{max} (expressed in mg of protein adsorbed per unit volume or weight of adsorbent) is calculated. The maximum volume or concentration of sample which can be applied per unit volume of adsorbent can then be determined. As mentioned earlier, the total bed volume required then depends on the method of purification (*Table 7*).

Separation based on structure

Method Table 1. General procedure for column packing.

1.	If necessary, pre-treat the matrix to allow removal of contaminants or to ensure pore swelling. Fibrous celluloses, for example, require pre-soaking in 0.5 M NaOH for 30 min while synthetic resins may need an alkali/acid cycle. Manufacturers' literature should be consulted.
2.	Remove any fines from the matrix. Mix the adsorbent with 5–6 times its volume of buffer, allow to settle and decant off the cloudy supernatant. Repeat 3–5 times.
3.	Remove the buffer until its volume is half that of the adsorbent; this ratio is generally most suitable for column packing. If necessary degas the slurry under suction.
4.	Clean the column, clamp the outlet and pour the matrix suspension in slowly with the column slightly tilted. An extension tube may be used if necessary. Packing can be speeded by opening the column outlet.
5.	Ensure the column is vertical, and filled to the top with buffer so that an upward meniscus appears, eliminating any air. Fit a column adaptor at the inlet and pump through at least two bed volumes of buffer prior to sample application.

4.2.3 *Column packing*

A general procedure for column packing is shown in *Method Table 1*. It is essential to remove any fines from the matrix prior to use. If these are not removed they will fill the voids between matrix particles and block the support thus reducing the flow rate and causing a non-uniform flow. The time required for matrix settling in fines removal depends on the adsorbent and can vary from a few minutes (e.g. resins) to half an hour in the case of cellulose.

The ratio of buffer to matrix used in column packing should provide a suspension which is sufficiently dilute to allow the escape of trapped bubbles, but not so thin that turbulence is promoted during packing, leading to particle distribution according to size. This is critical when using hydrogels but with more dense packings it is frequently possible to fluidize the bed by back-washing after packing in order to achieve an even bed.

An extension tube is supplied by some companies (e.g. Pharmacia) and is fitted to the column inlet during packing to allow all the matrix slurry to be poured at once. Extension tubes are usually of the same diameter as the column but half the length. Once a column is packed it should never be allowed to dry; this is particularly important in the use of hydrogels such as agarose where the matrix is irreversibly damaged upon drying.

4.2.4 *Sample application*

The introduction of the sample in low pressure separations can be achieved by stopping the flow of buffer and layering the sample directly onto the matrix bed. This method is described in *Method Table 2*.

The protein sample should be equilibrated in the same buffer used for column equilibration. This minimizes localized pH or ionic strength fluctuations which may

Method Table 2. Procedure for

1.	Drain off excess buffer fro of the bed.
2.	Close the outlet and layer so as to minimize disturbir
3.	Open the outlet and drain
4.	Apply elution buffer until it be washed in the process.

impair resolution. Samples should using centrifugation or precipitati

The sample may alternatively be to achieve this the sample density

The volume of sample applied dependent on the protein concentra rapid adsorption of protein to the c fluctuation through rapid counter-i column separations are usually best a protein concentration of 10–20 samples can be pumped directly

4.2.5 *Elution*

After sample application the colun unbound contaminants. For protei volume should be minimized in c

In column chromatography the p by adsorbing the protein follow contaminants, allowing the protei former option is most commonly degree of protein fractionation d

Elution of proteins from colum

(i) Isocratic elution—the compc of elution.

(ii) Stepwise elution—the elue conditions more favourable

(iii) Gradient elution—the eluen favouring protein dissociat

In isocratic elution the degree of position. A weak eluent may ther excessively strong eluent would le required for its success. Its chief ad for equipment and handling. It is s

acking.

oval of contaminants or to ensure
le, require pre-soaking in 0.5 M
may need an alkali/acid cycle.
l.
orbent with 5–6 times its volume
ly supernatant. Repeat 3–5 times.
iat of the adsorbent; this ratio is
necessary degas the slurry under

the matrix suspension in slowly
tube may be used if necessary.
mn outlet.
top with buffer so that an upward
umn adaptor at the inlet and pump
rior to sample application.

n *Method Table 1*. It is essential
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s the column but half the length.
dry; this is particularly important
trix is irreversibly damaged upon

tions can be achieved by stopping
nto the matrix bed. This method

e same buffer used for column
strength fluctuations which may

Method Table 2. Procedure for open column sample application.

1. Drain off excess buffer from the column top until its level just reaches the top of the bed.
2. Close the outlet and layer on the sample using a syringe via the column wall so as to minimize disturbing the bed.
3. Open the outlet and drain the sample onto the bed.
4. Apply elution buffer until it is 1–2 cm above the bed. The column wall should be washed in the process. Connect the column inlet and begin elution.

impair resolution. Samples should also be freed of particulates, including precipitate, using centrifugation or precipitation.

The sample may alternatively be layered onto the column bed under a layer of eluent; to achieve this the sample density is increased, usually with sucrose (3).

The volume of sample applied will vary. In ion-exchange chromatography it is dependent on the protein concentration of the sample; if too high (>50 mg ml^{-1}), the rapid adsorption of protein to the column may provide a localized pH and ionic strength fluctuation through rapid counter-ion release resulting in loss of activity. Reproducible column separations are usually best achieved with dilute protein samples. In ion exchange a protein concentration of 10–20 mg ml^{-1} is recommended (8). Large volumes of samples can be pumped directly onto the column.

4.2.5 *Elution*

After sample application the column may be washed in equilibration buffer to remove unbound contaminants. For proteins with partition coefficients less than 1 the washing volume should be minimized in order to limit loss of protein in the column wash.

In column chromatography the protein of interest may be separated from contaminants by adsorbing the protein followed by selective desorption or by adsorption of contaminants, allowing the protein to pass through the column without retention. The former option is most commonly used in protein purification since it allows a greater degree of protein fractionation during the elution step.

Elution of proteins from columns is achieved using three possible methods.

(i) Isocratic elution—the composition of the eluent does not change during the course of elution.
(ii) Stepwise elution—the eluent composition is changed at least once to provide conditions more favourable to elution.
(iii) Gradient elution—the eluent composition is changed continuously to conditions favouring protein dissociation from the packing.

In isocratic elution the degree of resolution is invariable and fixed by the eluent composition. A weak eluent may therefore never effectively elute bound protein while an excessively strong eluent would lead to loss of resolution. Trial and error is therefore required for its success. Its chief advantage is its simplicity and the minimal requirements for equipment and handling. It is seldom used in LPLC due to the improved resolution

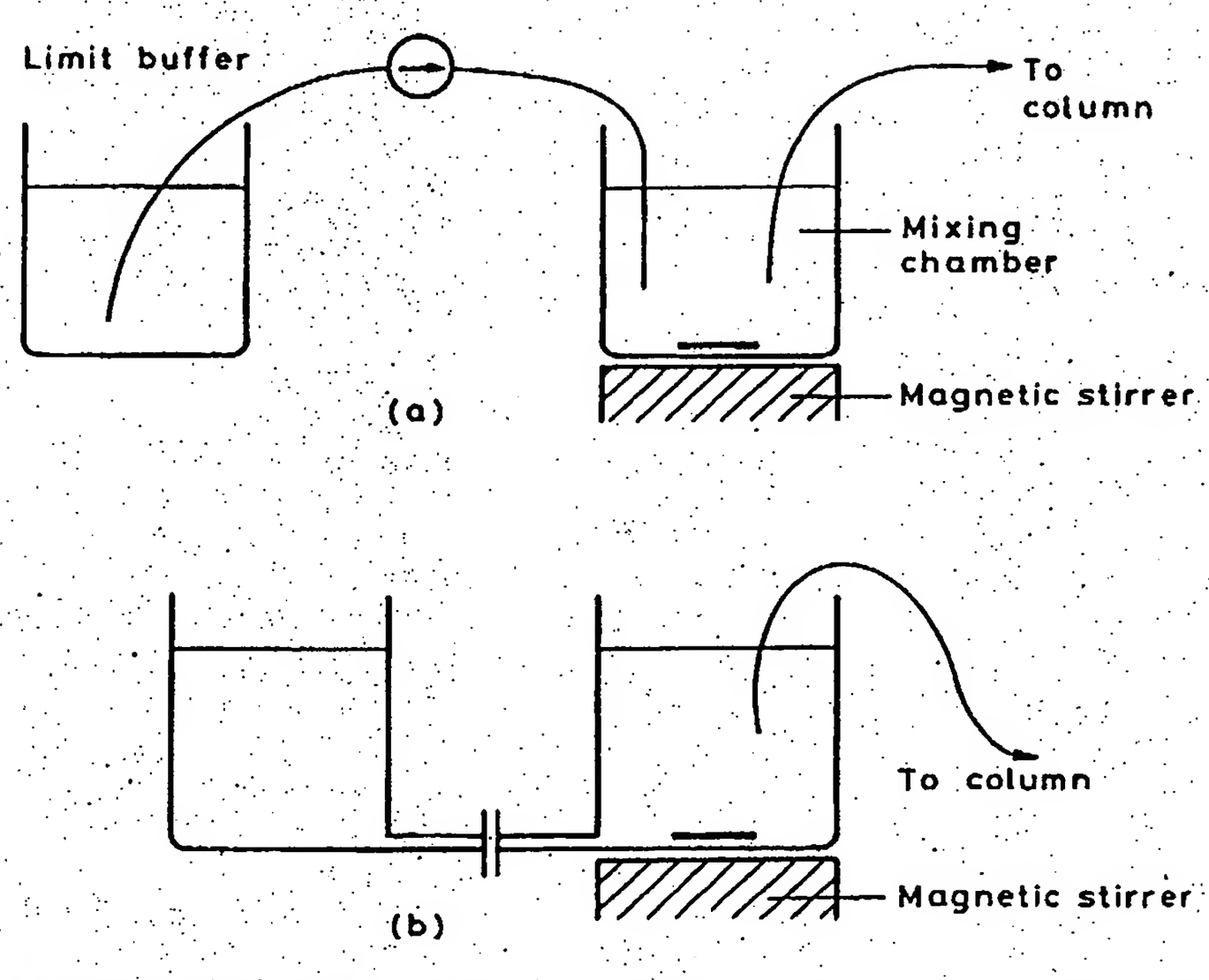


Figure 15. Simple apparatus for use in gradient elution consisting of two beakers connected via (a) a peristaltic pump or (b) a tube at their bases.

achievable using stepwise or gradient elution but is common in more rapid methods of protein resolution such as HPLC.

Stepwise elution is generally more reproducible than gradient methods (8) using laboratory apparatus. Two problems however arise in its use.

(i) Following a step change in elution conditions, a large number of proteins may be co-eluted with the consequence that selective desorption is lost.

(ii) The increase in partition coefficient which occurs as a protein is eluted may cause a fraction of the protein to remain bound to the column packing. Consequently in batch elution a protein may occur in several eluted fractions, while in column elution peak tailing may result.

Nevertheless stepwise elution is most commonly used in large-scale chromatography due to the simpler apparatus requirements compared to gradient methods. This elution method is also ideally suited to batch adsorption methods where the adsorbent is simply given a series of batch elution washes. Stepwise elution uses conditions which increasingly favour protein desorption from the adsorbent.

Gradient elution is the most widely used method of protein desorption in laboratory-scale chromatography. The changing elution conditions provide improved resolution over stepwise methods since peak tailing is not encouraged. On a laboratory scale a simple gradient elution apparatus can be constructed using two beakers joined at their

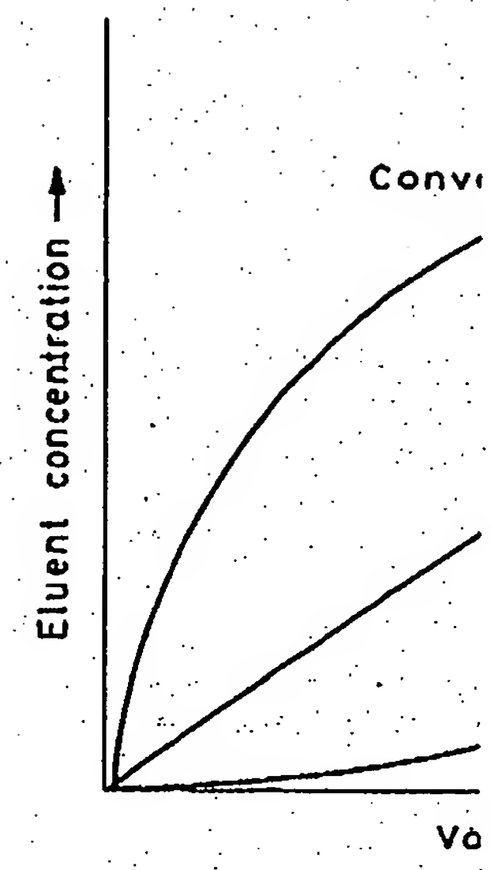


Figure 16. Alternative gradient shapes c

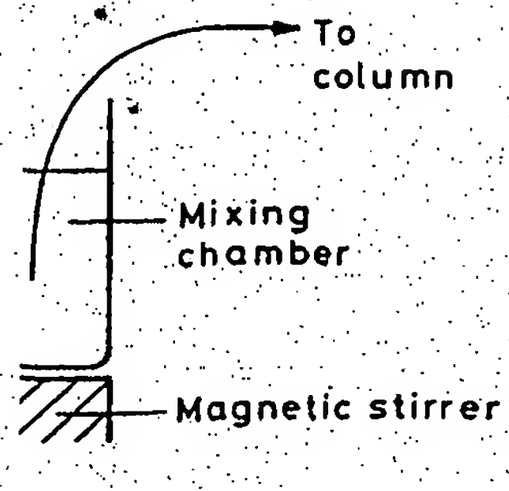
Table 8. The three basic types of gradie

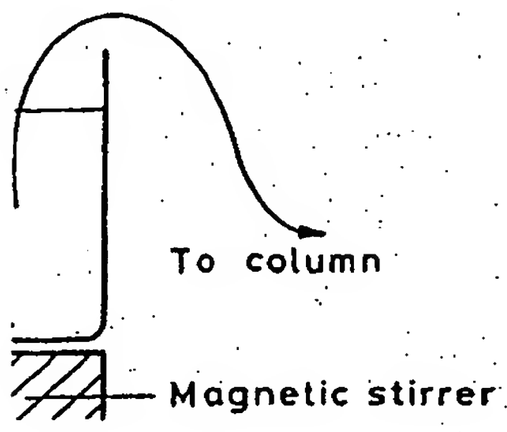
Form of gradient
Linear
Concave
Convex

R1, flow rate from limit buffer to mixin
R2, flow rate from mixing chamber to c

base or connected via a peristalt
chamber while the other determir
'limit' buffer. It is usually advisal
resolution is required, alter the gra
16). The steeper the gradient, the
is governed by the rate of mixing
gradient is produced by using twc
chamber at the same time as limit
as shown in *Table 8*.

In gradient elution the volume o
volumes may result in band spre
a wide window of solvent compo
two limit buffers are used or if the
Generally, however, the gradie
separations.





two beakers connected via (a) a peristaltic

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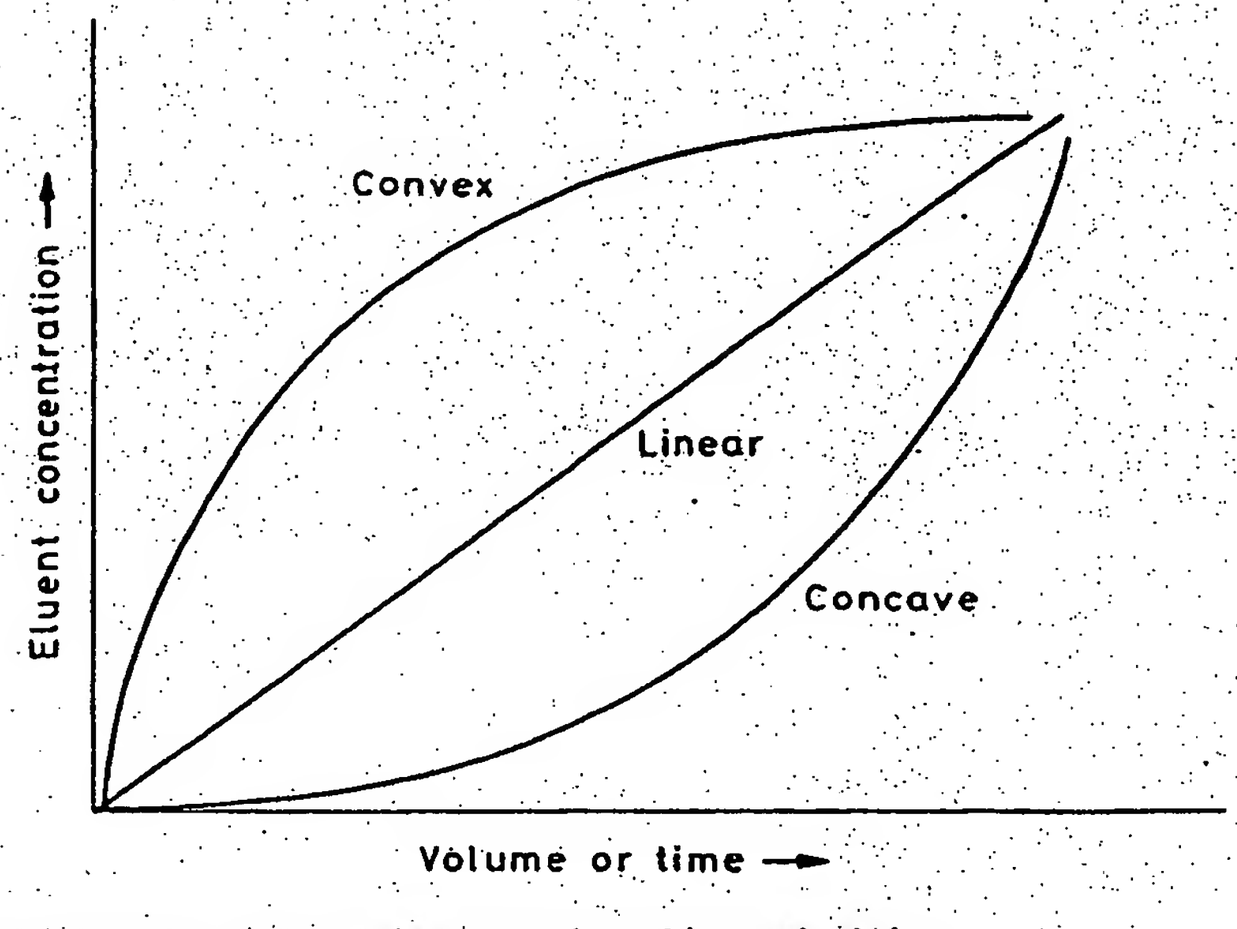

Figure 16. Alternative gradient shapes commonly used in protein elution.

Table 8. The three basic types of gradients used in column chromatography.

Form of gradient	*Requirement*	*Result*
Linear	$R_2 = 2R_1$	
Concave	$R_2 > 2R_1$	Better resolution initially
Convex	$R_2 = R_1$ or $R_2 < 2R_1$	Better resolution at end

R1, flow rate from limit buffer to mixing chamber.
R2, flow rate from mixing chamber to column.

base or connected via a peristaltic pump (*Figure 15*). One beaker acts as a mixing chamber while the other determines the upper limits of the gradient and is termed the 'limit' buffer. It is usually advisable to use a linear gradient initially and then, if more resolution is required, alter the gradient shape either to a convex or concave shape (*Figure 16*). The steeper the gradient, the closer proteins will be eluted. The form of the gradient is governed by the rate of mixing the limit buffer with the mixing chamber. A linear gradient is produced by using two pump channels. Buffer is removed from the mixing chamber at the same time as limit buffer is pumped in. The gradient shapes are produced as shown in *Table 8*.

In gradient elution the volume of eluent should be about five bed volumes (16). Larger volumes may result in band spreading and dilution since proteins will be eluted over a wide window of solvent composition. More complex gradients may be produced if two limit buffers are used or if the strength of the limit buffer is altered during elution. Generally, however, the gradient shapes described above are sufficient for most separations.

4.2.6 *Flow rate selection*

The optimum flow rate for a separation is always a compromise between achieving maximum resolution and reducing process time. Prolonged elution times resulting from excessively low flow rates may, however, result in band spreading through diffusion. Conversely, at a high flow rate, separation times are faster but back mixing limits the resolution obtainable. While some packing materials may allow very fast flow rates with minimal back pressure, mass transfer restrictions may limit the equilibration between the pores and the interstitial space. Thus the optimum flow rate is considerably less than the maximum. The nature of the sample, particularly its viscosity, may also restrict the choice of flow rate. An estimate of the appropriate flow rate is usually found in the manufacturer's literature and may then be followed by laboratory-scale studies to determine the influence of increasing flow rate on resolution. Protein purifications are usually performed at a flow rate of 10–30 ml cm^{-2} h^{-1}. Some matrices (e.g. Sepharose Fast Flow) can be used at higher flow rates; however, flow rates used during fractionation should be much lower (20% of maximum value) to obtain optimum resolution. Higher flow rates may however be used during sample loading, washing and buffer re-equilibration stages. This is particularly useful in concentration steps where large volumes are processed.

A detailed discussion of the principles involved in column scale-up is outside the scope of this chapter. However, prior to scale-up the optimum linear flow rate (expressed as flow rate per unit of column cross-sectional area) and bed height are determined on a small scale. On scale-up the bed diameter is then increased and the same linear flow rate used (18,19). In addition the volumes used in sample loading, washing and elution are increased in proportion to the increase in bed volume. The object of scale-up of a chromatographic separation is to obtain the same percentage yield and product quality in the same time (20).

4.2.7 *Column regeneration*

Following elution of the desired protein, strongly bound protein and non-protein material should always be removed from the adsorbent to prevent a slow build-up of contamination which would result in column fouling, loss of resolution, blockage and contamination of the purified sample.

In column chromatography regeneration is frequently achieved *in situ*. The conditions used depend on the chemical stability of the matrix in use, the nature of the contamination and the application. Hydrogel based ion exchangers, for example, are commonly regenerated in a high salt concentration (e.g. 1 M NaCl) to remove bound protein. This is followed by washing in one column volume of 0.5 M NaOH to remove the very strongly bound material. This alkali treatment also serves to sterilize the column packing and remove any lipids and pyrogens. The alkali must be washed out from the column using several column volumes of equilibration buffer so as to prevent matrix deterioration.

More strongly bound proteins may be removed using 6 M urea or detergents provided they are washed free from the column after use. Pharmacia-LKB recommend detergents are removed using an increasing ethanol gradient (25–95%) followed by butanol, ethanol and distilled water.

More chemically stable matrices such as the synthetic resins can withstand stronger regenerating conditions such as cy
should always be consulted.

4.2.8 *Storage*

Prolonged storage of the matrix
growth using the matrix as a sul
ethanol and, in non-therapeutic a
0.02% sodium azide or 0.5% ch
conditions should be avoided to p
details for regeneration of specif

4.3 Medium and high pressure

Both MPLC and HPLC equipme
successful use of LPLC.

(i) The pump must provide c
(ii) The column must be capal
(iii) The detector should have
through in a matter of sec

In MPLC, equipment has been d
the use of halide-containing buf
commonly used in HPLC have bee
The requirements of each compo

4.3.1 *Pumps*

A variety of pumps exist for use in
are designed on the common featur
operation of a ball-type valve cont
may direct solvent from two plung
plunger with two pump heads (e.g.

Table 9. List of some commonly used H

Manufacturer	*Nar*
Beckman	110
Bio-Rad	135
Cecil	CE
Gilson	302
LKB	215
Perkin Elmer	Seri
Pharmacia	P50
	P35
Philips	PU4
Shimadzu	LC-
Waters	501

regenerating conditions such as cycling in 1 M NaOH and acid. Manufacturers' literature should always be consulted.

4.2.8 *Storage*

Prolonged storage of the matrix requires the addition of agents to prevent microbial growth using the matrix as a substrate. Commonly used preservatives include 20% ethanol and, in non-therapeutic applications (9), 0.2% merthiolate, 0.02% hibitane, 0.02% sodium azide or 0.5% chloretone. Long term storage of matrices in alkaline conditions should be avoided to prevent degradation of the matrix structure. Additional details for regeneration of specific adsorbents are given in later sections.

4.3 Medium and high pressure equipment

Both MPLC and HPLC equipment have certain requirements not necessary for the successful use of LPLC.

(i) The pump must provide constant, pulse-free flow at increased back pressure.
(ii) The column must be capable of withstanding the increased pressure.
(iii) The detector should have a fast response time since protein peaks may pass through in a matter of seconds.

In MPLC, equipment has been designed to minimize protein denaturation and allow the use of halide-containing buffers (21). Consequently stainless steel components commonly used in HPLC have been replaced with borosilicate glass, titanium and teflon. The requirements of each component are discussed below.

4.3.1 *Pumps*

A variety of pumps exist for use in high performance separations (*Table 9*). The majority are designed on the common feature of one or two plunger pumps providing flow through operation of a ball-type valve controlling alternate solvent delivery and suction. Pumps may direct solvent from two plungers (e.g. Waters 501, LKB 2150) or by using a single plunger with two pump heads (e.g. Gilson 302, Beckman 110B). Where salt-containing

Table 9. List of some commonly used HPLC/MPLC pumps.

Manufacturer	*Name*	*Description*
Beckman	110B	Single piston
Bio-Rad	1350 Soft Start	Dual pistons
Cecil	CE 1100	Dual overlapping pistons
Gilson	302/303	Single piston
LKB	2150	Dual piston
Perkin Elmer	Series 100	Single piston
Pharmacia	P500	Biocompatible dual piston (FPLC)
	P3500	Biocompatible dual piston (HPLC)
Philips	PU4100	Dual piston
Shimadzu	LC-6A	Single piston
Waters	501	Dual piston

Separation based on structure

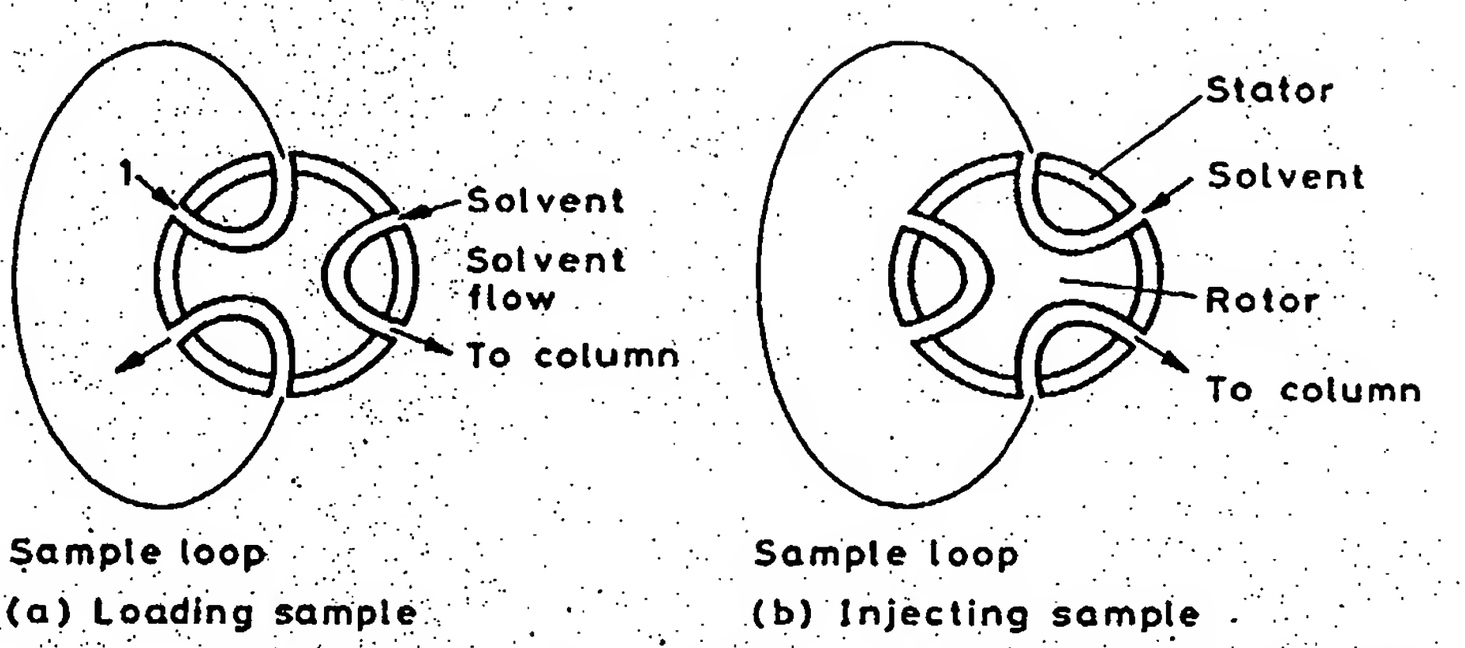

Figure 17. Loop injector design. The sample is first injected into a fixed volume loop (port 1) which is then incorporated into the solvent flow by rotating the inner cylinder.

buffers need to be used frequently stainless steel must be eliminated from all parts of the pump which come into contact with the fluid. In the Pharmacia P500 pump for example, a two plunger system is used consisting of borosilicate glass cylinders and fluoroplastic reinforced titanium plungers.

4.3.2 *Sample application*

In high performance separations a small volume of sample (10−100 μl) is usually injected into the solvent flow without interruption. This may be achieved using a Hamilton syringe directly to inject the sample through a hermetically sealing septum into the solvent flow. Loop injectors are however more common (e.g. Rheodyne) allowing the injection of a fixed volume of sample (e.g. 20 μl) into a sample loop which is then flushed through with solvent to inject the sample onto the column (*Figure 17*).

4.3.3 *Columns*

In HPLC the column and tubing must withstand the high operating pressure. Consequently stainless steel is used and sealing between the two is ensured by using metal ferrules compressed against steel nuts. Stainless steel columns are usually 15−30 cm long with an internal diameter of 2−9.5 mm, with 3.9 and 4.6 mm being most common. The trend towards the use of microbore systems for more rapid analysis using less solvent has, however, led to the use of even narrower and shorter column dimensions. Pre-packed columns are recommended for optimum resolution. In bio-compatible systems the elimination of stainless steel has been achieved through the use of borosilicate glass columns and, usually, teflon tubing.

Guard columns are recommended for high performance separations. These remove strongly adsorbed molecules so that fouling of the column head is prevented through use of these renewable inline pre-columns.

4.3.4 *Detectors*

The principle of detection used in high performance separation differs little from that

Table 10. List of some commonly us

Manufacturer	*Name*
Beckman	160 163 164
Cecil	CE 1200
Gilson	111B 116 HM/Holochrc
LKB	2151
Perkin Elmer	LC-90
Pharmacia	UVI UVM
Philips	PU 4100

used in low pressure chromatog
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adjustment. Most HPLC detect
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detectors. Some detectors allo
a spectrum analysis facility all
(e.g. the Philips PU4100). Hen
applications and have expensiv
purification. The choice will th

4.4 Basic procedures in HPL

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in protein purification.

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and a vacuum filter app

(ii) In order to prevent back
should be freed of pre
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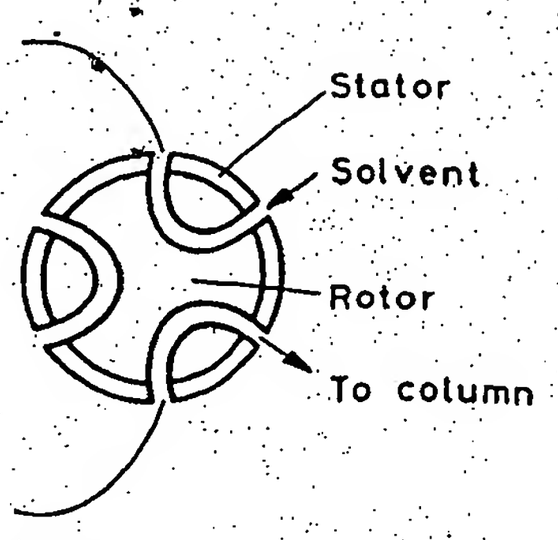
(iii) A low volume pre-colu
adsorbing components f



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f sample (10–100 μl) is usually . This may be achieved using a gh a hermetically sealing septum more common (e.g. Rheodyne) g. 20 μl) into a sample loop which ıple onto the column (*Figure 17*).

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nance separations. These remove olumn head is prevented through

separation differs little from that

Table 10. List of some commonly used HPLC/MPLC UV detectors.

Manufacturer	*Name*	*Description*
Beckman	160	Fixed wavelength 214–546 nm
	163	Variable wavelength 195–350 nm
	164	Variable wavelength 190–700 nm
Cecil	CE 1200	Variable wavelength 190–400 or 380–600 nm depending on lamp used
Gilson	111B	Fixed wavelength 254 or 280 nm
	116	Variable wavelength 190–380 nm Spectrum analysis
	HM/Holochrome	Variable wavelength 190–600 nm
LKB	2151	Variable wavelength 190–600 nm
Perkin Elmer	LC-90	Variable wavelength 195–390 nm
Pharmacia	UVI	Fixed wavelength for FPLC
	UVM	Fixed wavelength for FPLC
Philips	PU 4100	Variable wavelength 190–700 nm. Scanning accessory.

used in low pressure chromatography. The rapid separation time means, however, that the speed of response of high performance detectors should be faster. UV detectors are most commonly used in protein separations and although a fixed wavelength (e.g. 280 nm) is usually needed, many detectors are fitted with a variable wavelength adjustment. Most HPLC detectors (*Table 10*) are high cost and consequently fitted with a deuterium lamp in preference to the mercury lamps commonly used in low pressure detectors. Some detectors allow the analysis of two wavelengths concurrently or have a spectrum analysis facility allowing the spectral analysis of any chromatogram peak (e.g. the Philips PU4100). Hence many detectors are suitable in a wide range of HPLC applications and have expensive facilities not necessarily required for routine protein purification. The choice will therefore depend on the dedication of the system in use.

4.4 Basic procedures in HPLC/MPLC

The basic procedure for HPLC separations is given in *Method Table 3*. The following points should, in particular, be noted for the optimal use of both HPLC and FPLC in protein purification.

(i) In order to prevent gas bubbles forming and occluding the tubes the mobile phase should be de-gassed and filtered using 0.45 μm filters (e.g. Millipore, Whatman) and a vacuum filter apparatus.

(ii) In order to prevent back pressure increase due to particulate fouling, the sample should be freed of precipitate and suspended solids using centrifugation or filtration. It should not form a precipitate when added to the mobile phase.

(iii) A low volume pre-column should be used to remove particulates and strongly adsorbing components from the sample, thereby prolonging column life. The

Separation based on structure

Method Table 3. General procedure for protein purification using high performance techniques.

1. Degas and filter the mobile phase into a suitable bottle using a 0.45-μm filter and vacuum pump.
2. Put the pump inlet line into the mobile phase reservoir and draw the liquid along the tubing line using a syringe.
3. Ensure no air is trapped in the pump heads or pre-column tubing by disconnecting the column inlet and running the pump for a few minutes. This is termed 'Priming'. Stop the pump.
4. Connect up the column inlet and start the pump. A flow rate of 1 ml min^{-1} is common on an analytical scale. Run the pump for several minutes until the column is equilibrated. This is usually evident in a stable detector output.
5. Inject the sample. Sample volumes of up to 200 μl are usual on an analytical scale for HPLC but may be 2–5 ml using a 1 ml FPLC column.
6. Separation is commonly achieved using isocratic or gradient elution, typical separations last up to 1 h.
7. Optimize the degree of resolution further if necessary by adjusting the mobile phase composition or the gradient shape.
8. Prior to injecting another sample re-equilibrate the column to the initial running conditions.

pre-column or, if refillable, its packing, should be replaced as required by the application. Crude samples will require frequent pre-filter replacement. Deterioration of peak shape and resolution and increased pressure mean the pre-column should be replaced.

(iv) Solvents used in purification should be high quality (HPLC Grade). Manufacturers include Waters and Fisons. They should not interfere with protein detection and the use of low-UV grade solvents is often practised when detecting at the lower wavelengths. Many are toxic (e.g. acetonitrile and tetrahydrofuran) and care should be exercised in their handling and subsequent disposal.

(v) Caution should be exercised in the use of halide containing mobile phases in direct contact with metal equipment components. The resulting corrosion and abrasive effect of salt crystals may limit equipment life. The equipment should be thoroughly washed out with a non-halide-containing mobile phase after their use.

(vi) Silica based packings should not be used routinely above pH 8. The use of chelating agents with silicas should also be avoided and in the choice of column packings, 'end-capped' silicas are preferred. End-capping minimizes the presence of unreacted silanol groups which may cause unwanted interference effects during a separation.

5. SEPARATION ON THE BASIS OF CHARGE

Ion-exchange is the most commonly practised chromatographic method of protein purification. This stems, in part, from its ease of use and scale-up, wide applicability

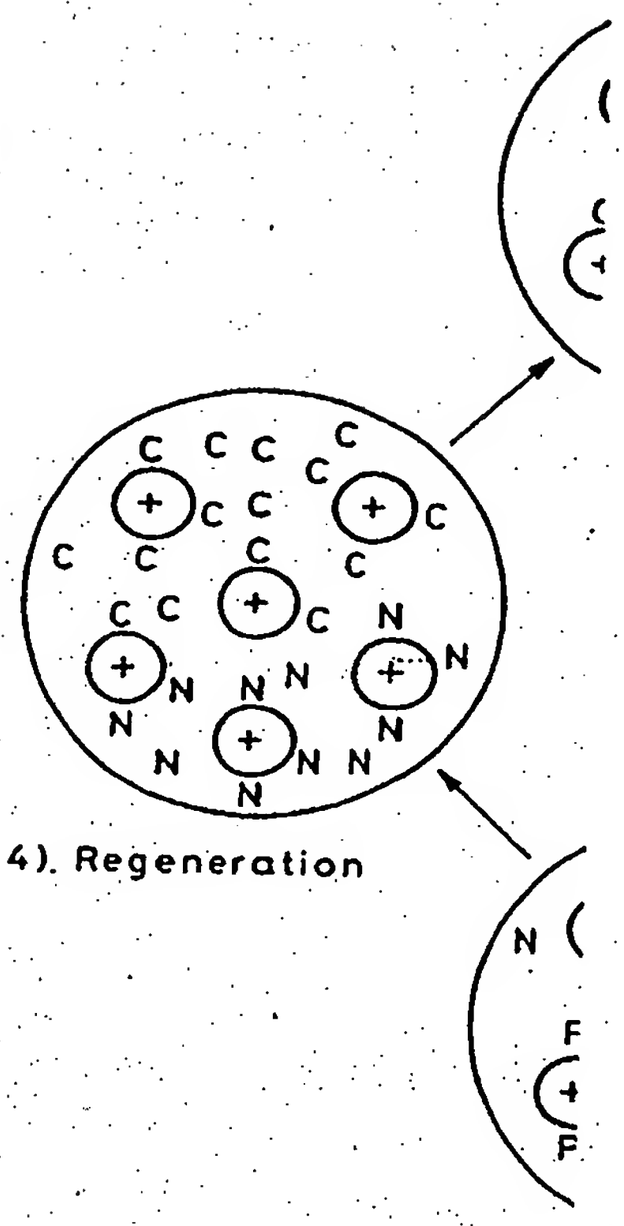

Figure 18. Diagramatic representation of i
Desorption and regeneration may be acc

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elution with fractionation and/or
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technique. For those seeking a m
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itable bottle using a 0.45-μm filter

reservoir and draw the liquid along

pre-column tubing by disconnecting
or a few minutes. This is termed

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200 μl are usual on an analytical
a 1 ml FPLC column.
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and scale-up, wide applicability

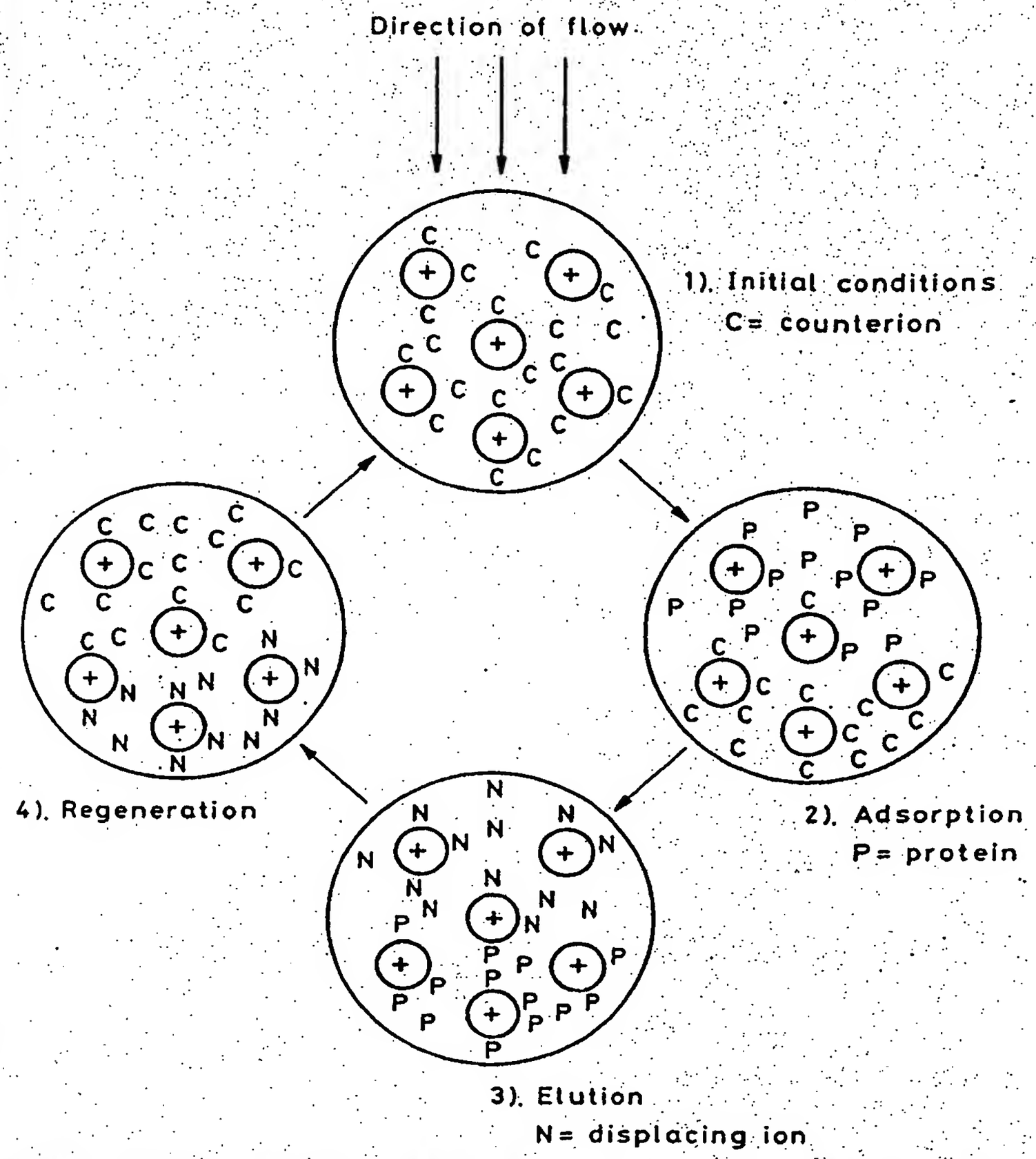


Figure 18. Diagramatic representation of the stages in the purification of a protein (P) using anion exchange. Desorption and regeneration may be accomplished in one step using the same counter-ion.

and low cost in comparison with other separation methods. Ion-exchange of proteins involves their adsorption to the charged groups of a solid support followed by their elution with fractionation and/or concentration in an aqueous buffer of higher ionic strength. The basic steps are illustrated in *Figure 18*. A brief introduction to ion-exchange theory will be presented in the next section followed by guidance on the use of this technique. For those seeking a more in-depth treatment of ion-exchange theory, the works of Vermeulen and co-workers (22), Scopes (8) and Osterman (3) are

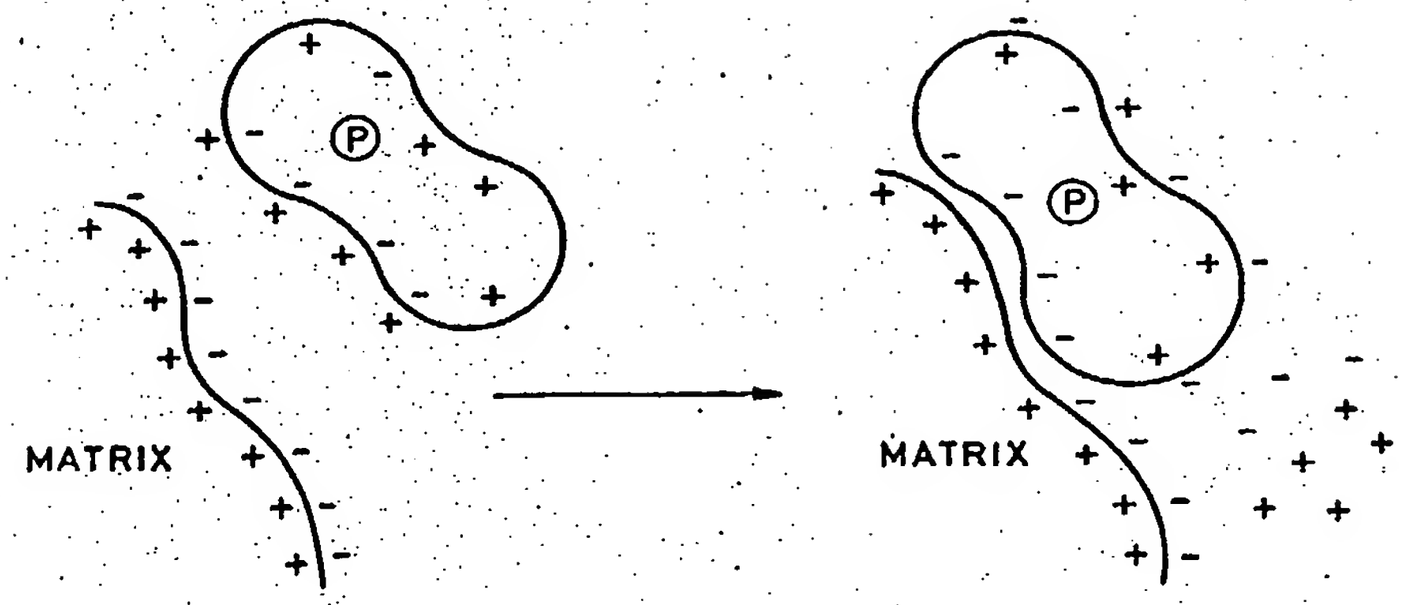


Figure 19. The dissociation of counter-ions occurs on adsorption of a protein (P). In this case multi-point binding has occurred, the adsorption is strong and a high counter-ion concentration may be required to promote elution.

recommended. Janson and Low (23), Scawen (10) and Chase (11) cover aspects of the large-scale use of ion-exchangers.

5.1 Theory of ion-exchange

Proteins carry both positive and negative charged groups on their surface, due largely to the side chains of acidic and basic amino acids. Positive charges are contributed by histidine, lysine, arginine and, to a lesser extent, N-terminal amines. Negative groups are due to aspartic and glutamic acids, C-terminal carboxyl groups and, to a lesser extent, cysteine residues. The net charge on a protein depends on the relative numbers of positive and negative charged groups; this varies with pH. The pH where a protein has an equal number of positive and negative charged groups is termed its isoelectric point (pI). Most proteins have a pI between pH 5 and 9. Above their pI proteins have a net negative charge while below it their overall charge is positive.

Ion-exchange is the separation of proteins on the basis of their charge and can be used to resolve proteins which differ only marginally in their charged groups. Separation of proteins is achieved by their difference in equilibrium distribution between a buffered mobile phase and a stationary phase consisting of a matrix to which charged inorganic groups are attached. For the effective use of ion-exchange in protein purification the stationary phase must therefore be capable of binding either positively-charged or negatively-charged proteins. To this end ion-exchange matrices are derivatized with positively-charged groups for the adsorption of anionic proteins (termed anion exchangers) or negatively-charged groups for the adsorption of cationic proteins (cation exchangers).

Associated with both stationary phase and protein charged groups are counter-ions which are simple, low molecular weight ions. In order for the protein to bind to the stationary phase, therefore, the counter-ions of both groups must become electrolytically dissociated (*Figure 19*).

Counter-ions 'screen' the exchanger groups, preventing their binding with a protein. Na^+ and H^+ are the common counter-ions for cation exchangers while Cl^- and OH^-

are usually used with cation excha
series (*Figure 20*) according to their
groups at equal concentration. Co
at equal strength as the counter-i
permanently bind to an ionogenic
shifting between the bulk solution ar
groups can become uncovered to all
ion concentration, the less frequentl
to the use of an ion exchanger the c
ion more suitable to the particular
is discussed in more detail in Sec

5.2 Selection of conditions for io

A wide variety of matrices, functi
are available. The factors involved
to guide the practitioner in their s

Cations: $Ag^+ > Cs^+ >$ I
Anions: $I^- > NO_3^- > PO_4^- > CN^- >$ HSO

Figure 20. The activity series of anionic a
each series have a stronger attraction for t

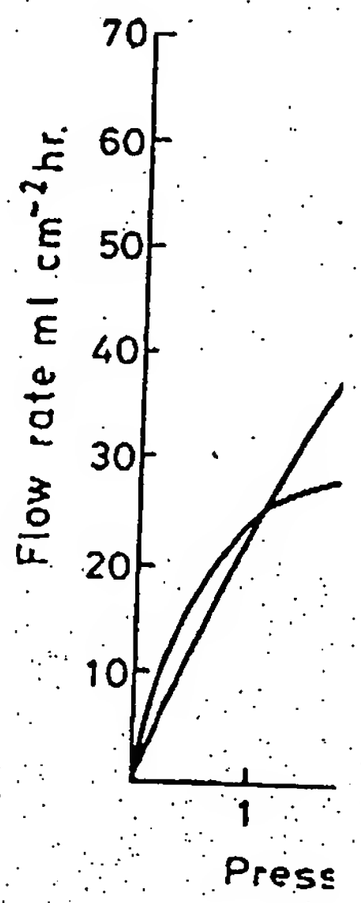
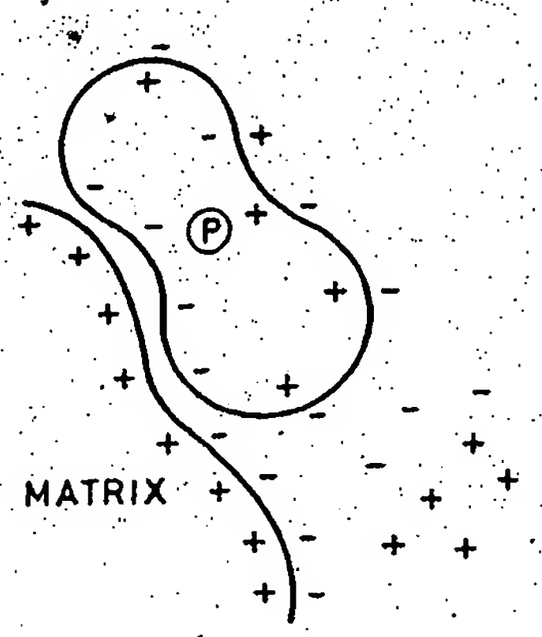


Figure 21. Variation in flow rate with pressu
2.5 × 45 cm, pH = 7, ionic strength = 0.



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) and Chase (11) cover aspects of

groups on their surface, due largely . Positive charges are contributed N-terminal amines. Negative groups l carboxyl groups and, to a lesser in depends on the relative numbers s with pH. The pH where a protein ged groups is termed its isoelectric nd 9. Above their pI proteins have charge is positive.

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n charged groups are counter-ions rder for the protein to bind to the groups must become electrolytically

enting their binding with a protein. on exchangers while Cl^- and OH^-

are usually used with cation exchangers. Counter-ions can be arranged in an 'activity' series (*Figure 20*) according to their strength of interaction with their respective ionogenic groups at equal concentration. Consequently chloride would replace hydroxide ions at equal strength as the counter-ion for an anion exchanger. Counter-ions do not permanently bind to an ionogenic group but stay in a state of equilibrium, continually shifting between the bulk solution and the exchanger groups. It follows that the ionogenic groups can become uncovered to allow the binding of a protein. The higher the counter-ion concentration, the less frequently do the ionogenic groups become uncovered. Prior to the use of an ion exchanger the counter-ion may require replacement with a different ion more suitable to the particular application. Matrix pre-treatment and conditioning is discussed in more detail in Section 5.3.

5.2 Selection of conditions for ion-exchange purifications

A wide variety of matrices, functional groups, and adsorption/desorption conditions are available. The factors involved in making each choice are described below so as to guide the practitioner in their selection.

Cations: $Ag^+ > Cs^+ > Rb^+ > K^+ \geq NH_4^+ > Na^+ > H^+ > Li^+$

Anions: $I^- > NO_3^- > PO_4^- > CN^- > HSO_3^- > Cl^- > HCO_3^- > HCOO^- > CH_3COO^- > OH^- > F^-$

Figure 20. The activity series of anionic and cationic counter-ions. The counter-ions at the beginning of each series have a stronger attraction for the ionogenic groups of the ion exchangers.

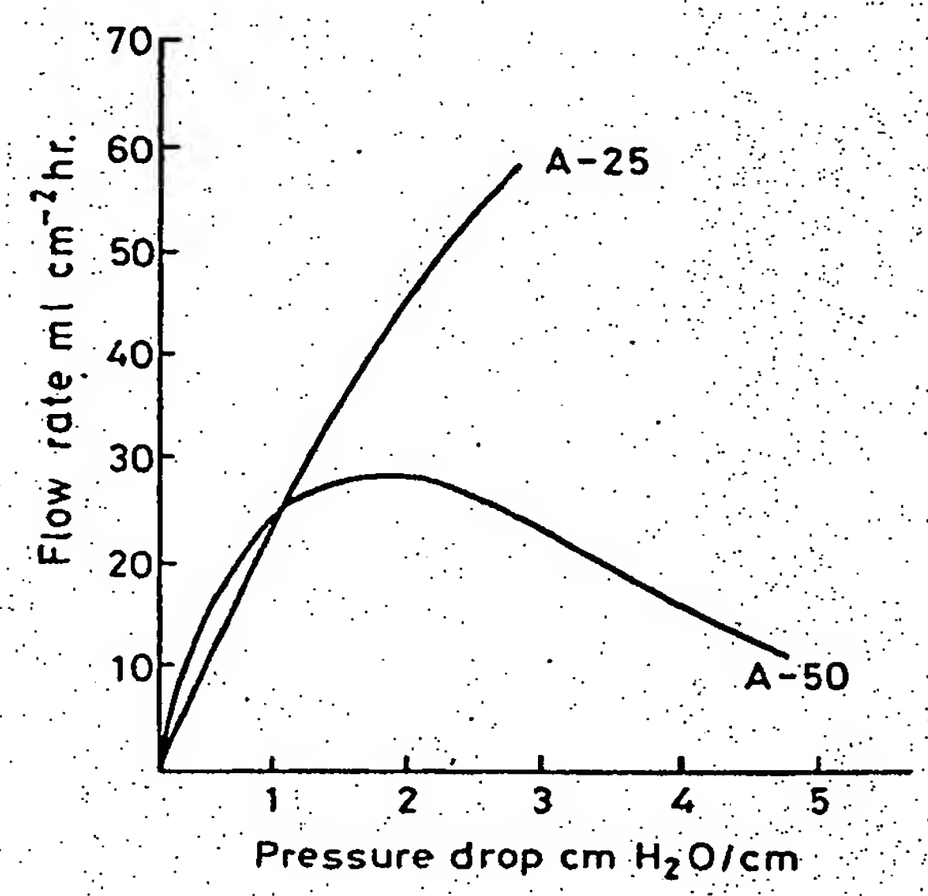


Figure 21. Variation in flow rate with pressure drop across a bed of DEAE–Sephadex. Bed dimensions = 2.5 × 45 cm, pH = 7, ionic strength = 0.1 M. Reproduced with kind permission of Pharmacia-LKB.

Table 11. List of commonly used ion exchangers.

Matrix	*Manufacturer/ Trade name*	*Particle size (μm)*	*pH range*	*Comments*
A. Low and medium pressure packings				
Cellulose				
Fibrous	Whatman			
	Bio-Rad Cellex	80 × 18		High flow rates (100 ml cm^{-2} h^{-1})
	Serva	50–200		but limited resolution—ideal for initial fractionation-capacities up to 100 mg g^{-1}. Require pre-cycling.
Microgranular	Serva	100–200		Flow rates 100 ml cm^{-2} h^{-1}
	Whatman			Improved resolution over fibrous.
Beaded	Pharmacia	40–160	2–9	Flow rates 100 ml cm^{-2} h^{-1}
	Serva	50–160		Improved resolution over fibrous. Exclusion limit 10^6.
Agarose	Pharmacia Sepharose	45–165	2–14	M^{ex} 4 × 10^6
	Sepharose Fast Flow	45–165		Usable at flow rate >300 ml cm^{-2} h^{-1}
Dextran	Pharmacia A/C 25	40–125	2–13	A/C usable only up to 30 ml cm^{-2} h^{-1}. Matrices swell and shrink depending on ionic strength.
Polyacrylate	IBF-Trisacryl	40–80	<–13	$M^{ex}>10^7$. Chemically stable.
	Bio-Rad Biorex 70	Up to 1180		M^{ex} 7 × 10^4
	Anachem-Separon	50–80		35 nm pores—suitable for HPLC.
Polyvinyl	Merck Fractogel-5	25–50	1–14	Suitable for MPLC—resistant to 7 bar.
Composites (Kieselguhr -Agarose)	Sterling Organics	160–1000	4–10	$M^{ex}>10^6$. Highly porous and good hydrodynamic properties.
Polystyrene-dvb	Bio-Rad	Up to 1180		Non-specific adsorption from hydrophobic matrix.
	BDH		1–14	
	Serva	Up to 1000		
Silica	Serva	40–200	2–9	$M^{ex}>10^6$. Suitable for MPLC. Good hydrodynamic properties. Not usable above pH 9.
Polyether	Pharmacia Mono Q	10	2–12	Used in HPLC (FPLC) system,
	Mono S	10	2–12	Max. pressure 750 p.s.i.
B. High pressure packings				
Silica	Alltech			
	Synchropak 300	6.5	<9	300 nm pores. Polymeric amine coated.

Dextran	Pharmacia A/C 25	40–125	2–13	A/C usable only up to 30 ml cm^{-2} h^{-1}. Matrices swell and shrink depending on ionic strength.
Polyacrylate	IBF-Trisacryl	40–80	<–13	$M^{ex} > 10^7$. Chemically stable.
	Bio-Rad Biorex 70	Up-to 1180		M^{ex} 7 × 10^4
	Anachem-Separon	50–80		35 nm pores—suitable for HPLC.
Polyvinyl	Merck Fractogel-5	25–50	1–14	Suitable for MPLC—resistant to 7 bar.
Composites (Kieselguhr -Agarose)	Sterling Organics	160–1000	4–10	$M^{ex} > 10^6$. Highly porous and good hydrodynamic properties.
Polystyrene-dvb	Bio-Rad	Up to 1180		Non-specific adsorption
	BDH		1–14	from hydrophobic matrix.
	Serva	Up to 1000		
Silica	Serva	40–200	2–9	$M^{ex} > 10^6$. Suitable for MPLC. Good hydrodynamic properties. Not usable above pH 9.
Polyether	Pharmacia Mono Q	10	2–12	Used in HPLC (FPLC) system,
	Mono S	10	2–12	Max. pressure 750 p.s.i.
B. High pressure packings				
Silica	Alltech Synchropak 300	6.5	<9	300 nm pores. Polymeric amine coated.
	Anachem Anagel TSK/IEC			Silica or resin based.
	Anachem Dynamax Hydropore	12		Polyethyleneimine coated.
	Bio-Rad Bio-S 300	5, 10	<9	Pores size up to 400 nm.
	Brownlee Aquapore	10		Polymeric amine coated.
Resins	Anachem Anagel TSK/IEC			
	Beckman Spherogel TSK			
	Bio-Rad TSK IEX	10–20	2.5–12	Hydroxylated polyether.
	Waters Protein-PAK	10		100 nm pores, hydrophilic polymer coated resin.

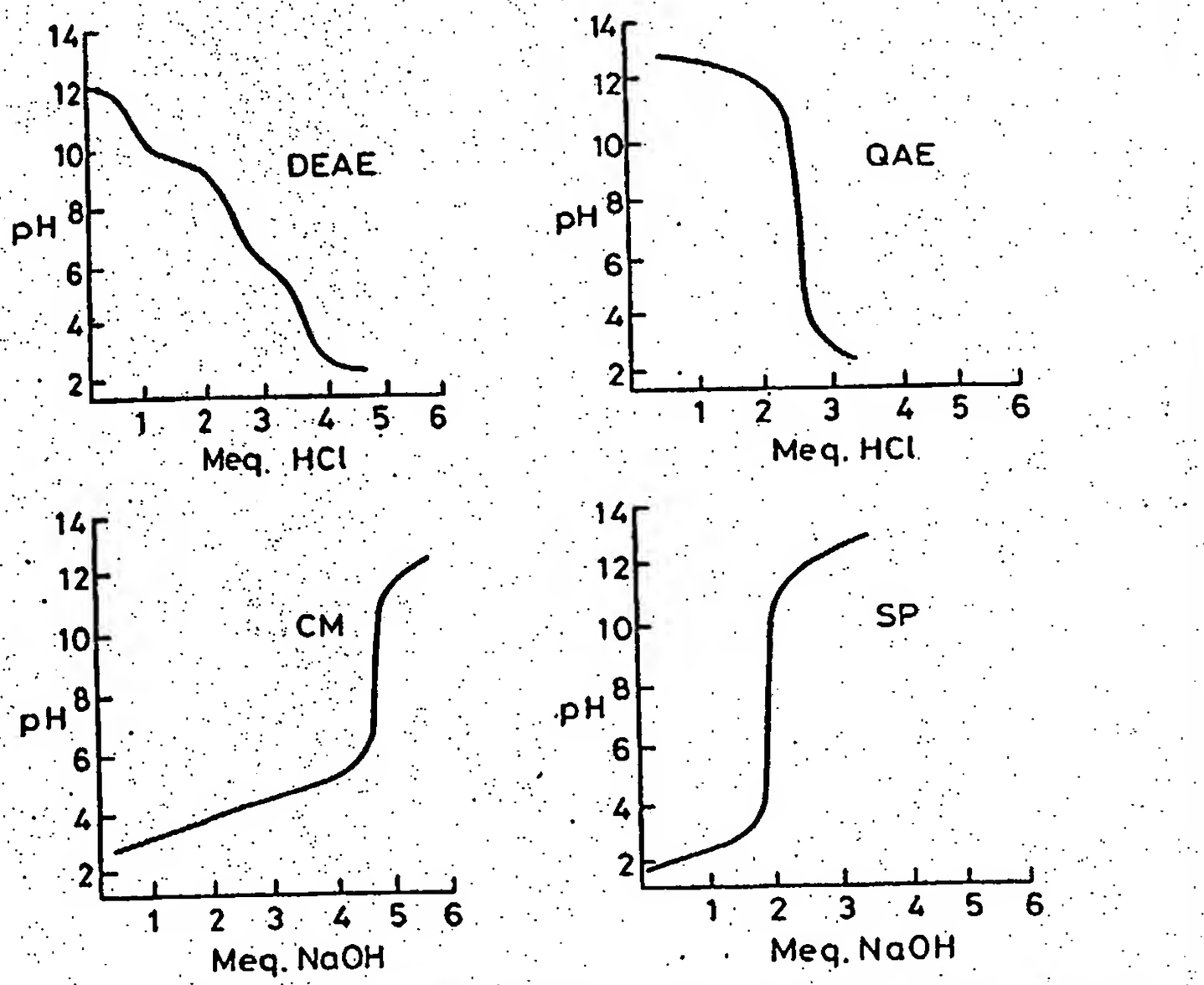


Figure 22. Typical titration curves for strong and weak anion and cation exchangers. The strong ion exchangers (QAE and SP groups) are fully ionized over the entire pH range used to purify proteins (3–11) while the weak ion exchangers (DEAE and CM) are ionized over a narrow pH range. Reproduced with kind permission from Pharmacia–LKB.

5.2.1 *Choice of matrix*

Section 3 should be consulted for guidance on the choice of support matrices. The characteristics of some of the more commonly used ion-exchange matrices (24,25) are summarized in *Table 11*. Related matrices can exhibit marked differences (see *Figure 21*), and manufacturers' literature should always be consulted.

5.2.2 *Selection of functional groups*

Ionogenic groups used in ion exchange are, as mentioned earlier, either positively-charged (anion exchangers), or negatively-charged (cation exchangers). Both types can be further divided into strong and weak groups. Strong ionogenic groups remain ionized over the whole operating pH normally used in protein purification while weak groups have a narrower effective pH range and are largely only partly ionized. Typical titration curves for both strong and weak ion exchangers are shown in *Figure 22*. The ionogenic groups used in the ion exchange of proteins are listed in *Table 12*, together with their structural formulae.

The most commonly used functionalities are the weak ionogenic groups. The

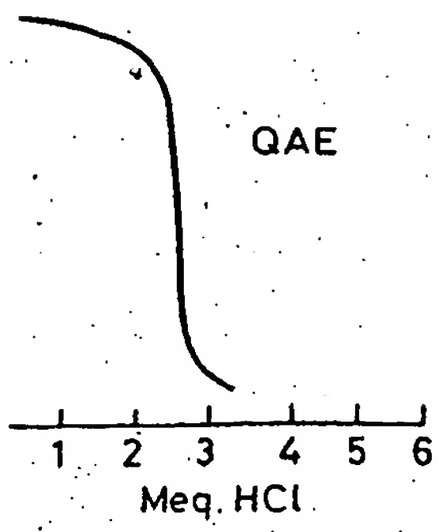
Table 12. Ion-exchange groups used in t

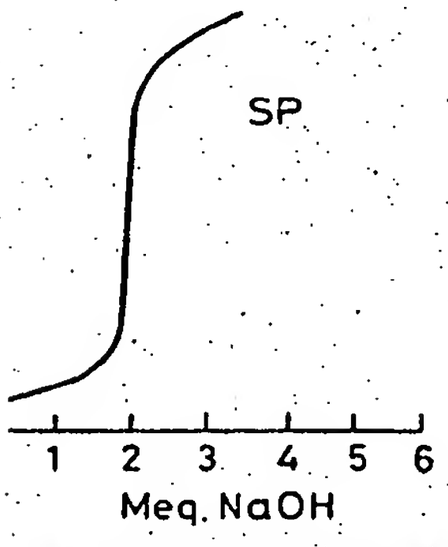
Formula
Strong anion
$-CH_2N^+(CH_3)_3$
$-C_2H_4N^+(C_2H_5)_3$
$-C_2H_4N^+(C_2H_5)_2CH_2CH(OH)CH_3$
Weak anion
$-C_2H_4N^+H_3$
$-C_2H_4NH(C_2H_5)_2$
Strong cation
$-SO_3-$
$-CH_2SO_3-$
$-C_3H_6SO_3-$
Weak cation
$-COO-$
$-CH_2COO-$

diethylaminoethyl (DEAE) group is charged proteins while the carbo: exchange for the recovery of pos becoming more popular in prote packings, based on sulphomethyl a and anion exchange, respectively.

In selecting the most suitable fu the desired protein should be con net positive or negative charge de the isoelectric point, respectively. an anion or cation exchanger. In pH stability of the protein. If a prote should be chosen. Conversely if t exchanger should be chosen. In pro DEAE) are most frequently used

The purification of α-amylase The pI of the enzyme is 5.2 so th stability of the enzyme falls off ra cation exchangers below pH 5 is th bearing an anion exchange functio isoelectric focusing or by referenc (26–28). According to Osterman on an ion-exchange matrix can be ionogenic groups will of course ha proteins of molecular weight 60– the opportunity to bind to several i and become uncovered by counter





ion exchangers. The strong ion exchangers used to purify proteins (3–11) while the I range. Reproduced with kind permission

choice of support matrices. The on-exchange matrices (24,25) are t marked differences (see *Figure* consulted.

tioned earlier, either positively- ition exchangers). Both types can ionogenic groups remain ionized purification while weak groups y partly ionized. Typical titration own in *Figure 22*. The ionogenic in *Table 12*, together with their

weak ionogenic groups. The

Table 12. Ion-exchange groups used in the purification of proteins.

Formula	*Name*	*Abbreviation*
Strong anion		
$-CH_2N^+(CH_3)_3$	Triethylaminoethyl	TAM–
$-C_2H_4N^+(C_2H_5)_3$	Triethylaminoethyl	TEAE–
$-C_2H_4N^+(C_2H_5)_2CH_2CH(OH)CH_3$	Diethyl-2-hydroxypropylaminoethyl	QAE–
Weak anion		
$-C_2H_4N^+H_3$	Aminoethyl	AE–
$-C_2H_4NH(C_2H_5)_2$	Diethylaminoethyl	DEAE–
Strong cation		
$-SO_3-$	Sulpho	S–
$-CH_2SO_3-$	Sulphomethyl	SM–
$-C_3H_6SO_3-$	Sulphopropyl	SP–
Weak cation		
$-COO-$	Carboxy	C–
$-CH_2COO-$	Carboxymethyl	CM–

diethylaminoethyl (DEAE) group is usually used in anion exchange to purify negatively-charged proteins while the carboxymethyl (CM) group is frequently used in cation exchange for the recovery of positively-charged groups. Strong ion exchangers are becoming more popular in protein purification, notably the Sepharose Fast Flow packings, based on sulphomethyl and triethylaminoethyl functionalities for strong cation and anion exchange, respectively.

In selecting the most suitable functional group for a purification the pH stability of the desired protein should be considered. Proteins are amphoteric; they may carry a net positive or negative charge depending on whether the buffer pH is below or above the isoelectric point, respectively. The choice must therefore be made between using an anion or cation exchanger. In practice the decision is sometimes restricted by the pH stability of the protein. If a protein is more stable above its pI then an anion exchanger should be chosen. Conversely if the protein is more stable below its pI then a cation exchanger should be chosen. In protein purification anion exchange functionalities (e.g. DEAE) are most frequently used since proteins of pI below 7 are more common.

The purification of α-amylase can be used as an example of exchanger selection. The pI of the enzyme is 5.2 so that weak ion exchangers can be used. However, the stability of the enzyme falls off rapidly below pH 5 (*Figure 23*). The effective use of cation exchangers below pH 5 is therefore prevented, restricting the choice to matrices bearing an anion exchange functionality. The pI of a protein can be determined using isoelectric focusing or by reference to lists of protein pI's to be found in the literature (26–28). According to Osterman (3), the average distance between ionogenic groups on an ion-exchange matrix can be as little as 1–3 nm. In highly porous matrices many ionogenic groups will of course have a much wider separation. It follows that globular proteins of molecular weight 60–100 kd, which have a diameter of 7–10 nm, have the opportunity to bind to several ionogenic groups at once, provided they are charged and become uncovered by counter-ions. With certain microporous resins the density

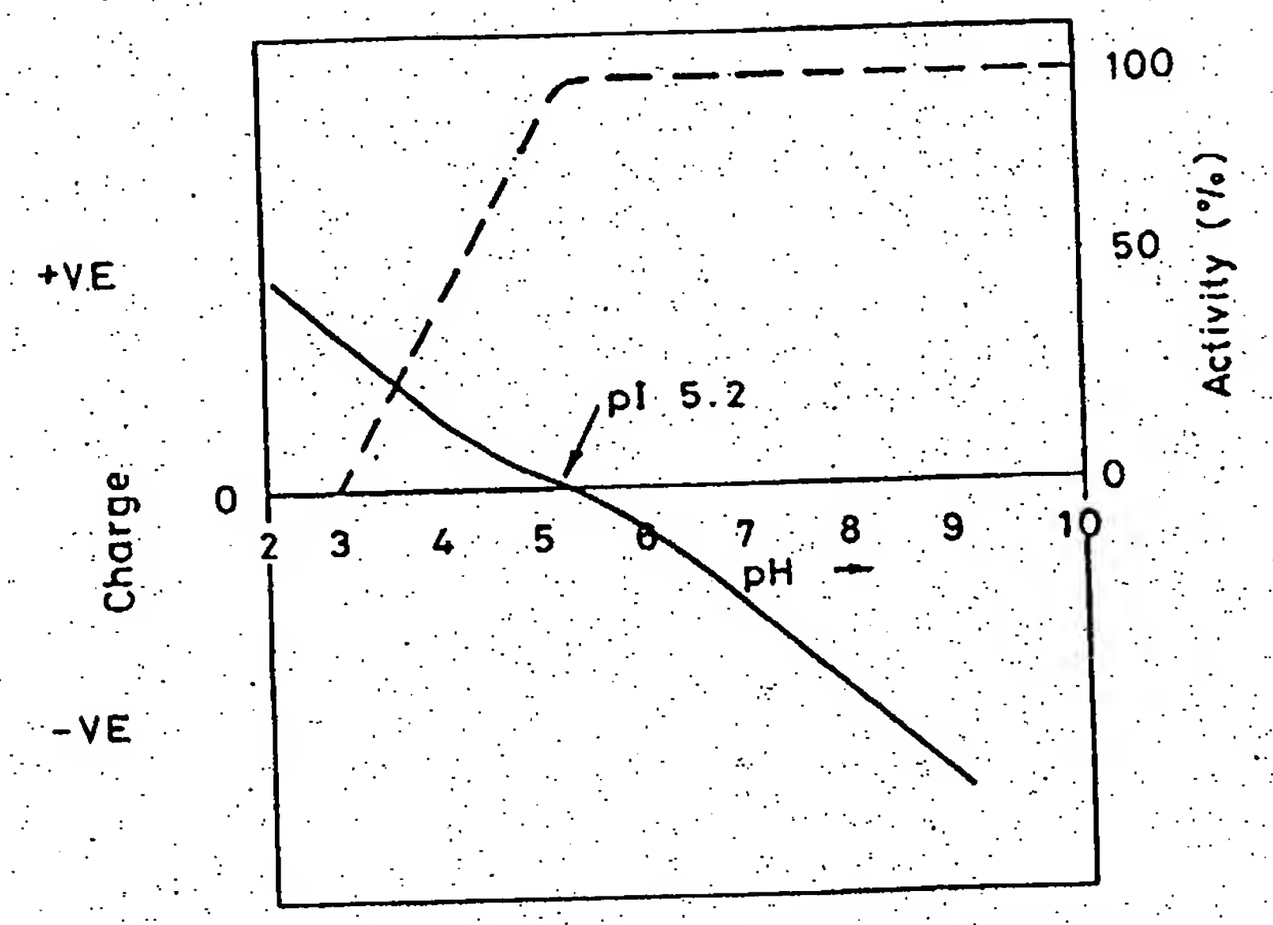


Figure 23. Simplified representation of pH stability (---) and net charge (—) of α-amylase. The isoelectric point of 5.2 prevents the effective use of cation exchangers.

of surface ionogenic groups is sufficiently high that multi-point protein adsorption can occur. This may result in the requirement for a high salt concentration (0.5 M NaCl) to promote elution; an 'all or nothing' adsorption elution pattern leading to minimal resolution, and even a possibility of protein denaturation. More porous supports have a less dense distribution of ionogenic groups so that the possibility of denaturation is minimal. With strong ion exchangers their ionogenic groups are all charged over a wide pH range (*Figure 22*). Consequently the chance for multi-point binding is higher than with weak ion exchangers. Where the aim of the ion exchange step is concentration this may be acceptable since a single, high concentration of salt is usually used for elution in a minimal volume and optimum resolution is not required. Weak ion exchangers however are normally chosen for the ion exchange of proteins between a pH of 6 and 9. They are recommended for the purification of labile proteins where mild eluting conditions are needed. Since weak cation exchangers lose their charge below pH 6 and weak anion exchangers above pH 9 (*Figure 22*), strong ion exchangers may be used for proteins of pI outside these pH values or for weakly-charged proteins which require an extreme pH to promote adsorption. Both strong and weak ion exchangers may therefore be used for protein adsorption. In the use of the former the increased potential for multi-point binding should be kept in mind with the result that stronger eluting conditions may be needed.

5.2.3 *Buffers used in ion exchange*

The mobile phase used in ion exchange is usually aqueous since the electrolyte properties of water contribute to the dissociation of the ionogenic groups and matrix swelling (8); both effects increase the rate of ion exchange.

Table 13. Buffers commonly used in the io

Ion exchanger	*Buffer*
Cation	Acetic
	Citric
	Mes
	Phosphate
	Hepes
Anion	L-Histidine
	Imidazole
	Triethanolai
	Tris
	Diethanolan

[a]See also Chapter 1.

In an aqueous solution the surfac counter-ions in a similar way to the i exchange of protein with the exchang strength may occur due to the release solution pH may also change (to a l in cation-exchange) causing protein ion exchange is always buffered to m the charge on a protein and its equilil minimum buffering strength recomm units of its pK value (8). The choice o minimizing pH fluctuation and maxi

Commonly used buffers in the ion most suitable buffer for a purificatio the adsorption pH (see also Chapter

Another reason for the use of buff pH differences which can result fro ion exchangers are frequently H^+ a cause localization of OH^- ions at the surface of cation exchangers, causing (*Figure 24*). This may result in an un protein denaturation. Consequently

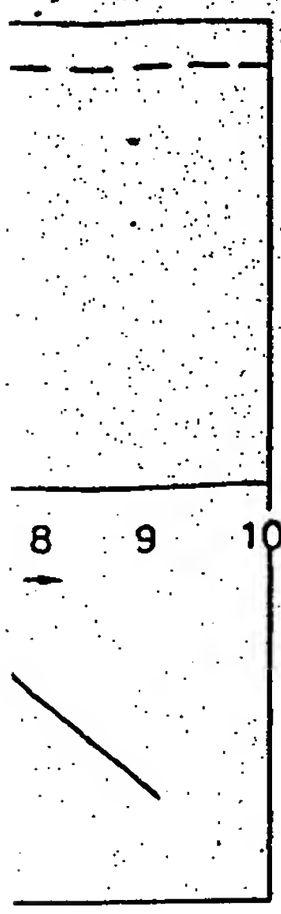
Lastly, two important rules shoul

(i) The charged form of the buffer (i.e. use negatively-charged b

(ii) The temperature will affect the will be different in purificatic

5.2.4 *Selection of adsorption and e*

The degree of ionic interaction betwe by adjusting the buffer pH to regulate ionogenic groups. The pH of the bu



harge (—) of α-amylase. The isoelectric

ulti-point protein adsorption can
salt concentration (0.5 M NaCl)
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ion. More porous supports have
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ication of labile proteins where
changers lose their charge below
22), strong ion exchangers may
r weakly-charged proteins which
trong and weak ion exchangers
use of the former the increased
ind with the result that stronger

ıs since the electrolyte properties
groups and matrix swelling (8);

Table 13. Buffers commonly used in the ion exchange of proteins[a].

Ion exchanger	*Buffer*	*pK*	*Buffering range*
Cation	Acetic	4.76	4.8–5.2
	Citric	4.76	4.2–5.2
	Mes	6.15	5.5–6.7
	Phosphate	7.20	6.7–7.6
	Hepes	7.55	7.6–8.2
Anion	L-Histidine	6.15	5.5–6.0
	Imidazole	7.0	6.6–7.1
	Triethanolamine	7.77	7.3–7.7
	Tris	8.16	7.5–8.0
	Diethanolamine	8.8	8.4–8.8

[a]See also Chapter 1.

In an aqueous solution the surface charge groups of a protein are associated with counter-ions in a similar way to the ionogenic groups on the exchanger. Following the exchange of protein with the exchanger-bound counter-ions an increase in solution ionic strength may occur due to the release of both protein and exchanger counter-ions. The solution pH may also change (to a lower pH in anion-exchange and to a higher pH in cation-exchange) causing protein denaturation. Consequently the mobile phase in ion exchange is always buffered to minimize pH fluctuations, which will also influence the charge on a protein and its equilibrium between stationary and mobile phases. The minimum buffering strength recommended for ion exchange is 10 mM within 0.3 pH units of its pK value (8). The choice of buffer strength is always a compromise between minimizing pH fluctuation and maximizing adsorbent capacity.

Commonly used buffers in the ion exchange of proteins are shown in *Table 13*. The most suitable buffer for a purification will depend on the choice of ion exchanger and the adsorption pH (see also Chapter 1).

Another reason for the use of buffers in ion exchange is to minimize the localized pH differences which can result from the Donnan effect. Since the counter-ions for ion exchangers are frequently H^+ and OH^-, the use of unbuffered solutions would cause localization of OH^- ions at the surface of anion exchangers and H^+ ions at the surface of cation exchangers, causing the Donnan effect which is a localized pH effect (*Figure 24*). This may result in an unpredictable ion-exchange process and even worse, protein denaturation. Consequently solutions are always buffered.

Lastly, two important rules should be remembered.

(i) The charged form of the buffer should not interfere with the ion-exchange process (i.e. use negatively-charged buffers such as acetate in cation exchange).

(ii) The temperature will affect the pK_a of the buffer such that its buffering capacity will be different in purifications carried out in the cold room.

5.2.4 *Selection of adsorption and elution pH*

The degree of ionic interaction between a protein and an ion exchanger can be controlled by adjusting the buffer pH to regulate the degree of ionization of both matrix and protein ionogenic groups. The pH of the buffer can therefore be adjusted in order to favour

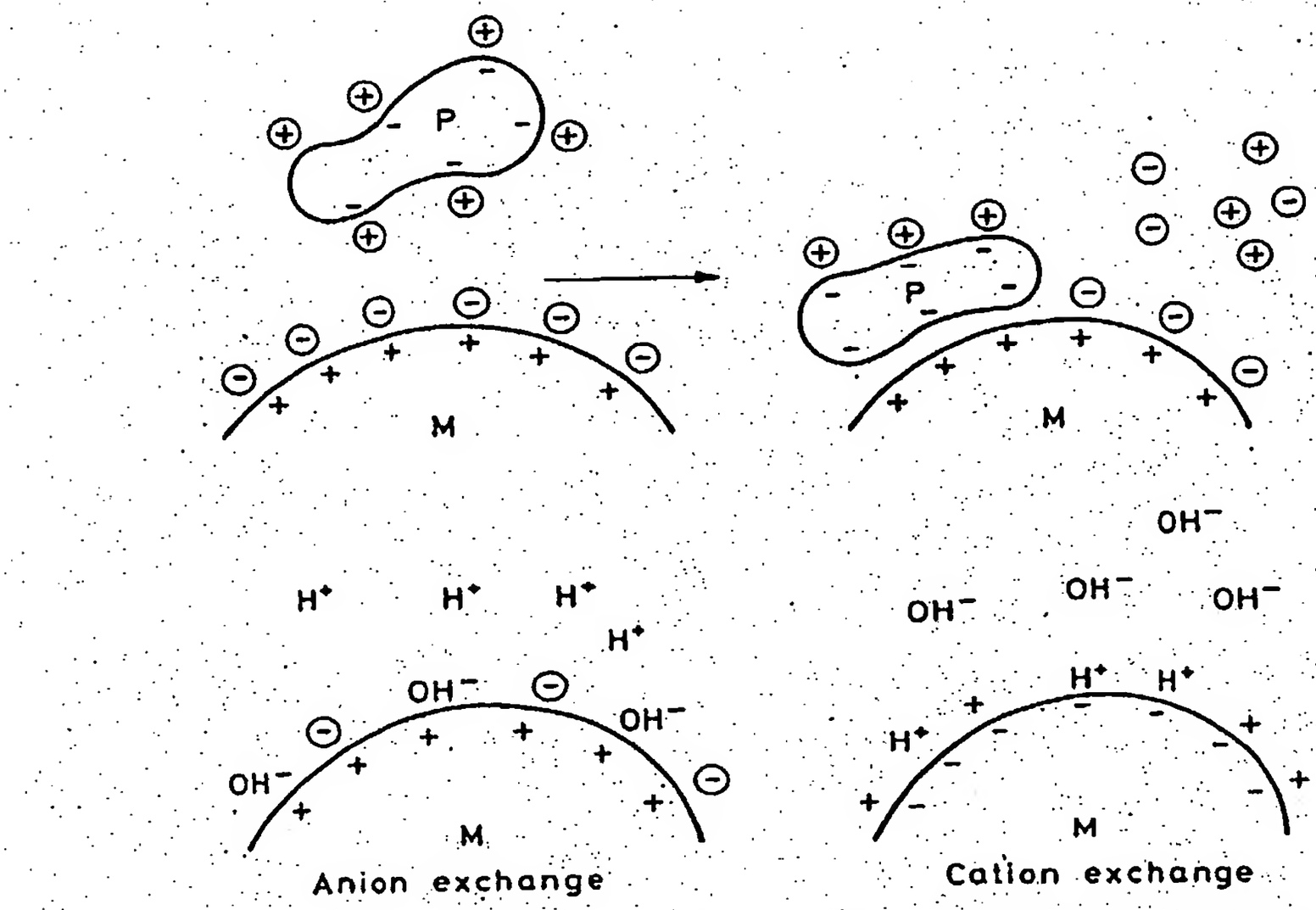


Figure 24. Donnan effect in anion- and cation-exchange which may cause localized pH differences in poorly buffered systems.

adsorption of protein to the ion exchanger or encourage protein–matrix dissociation and sample elution.

For protein adsorption a pH about 1 unit above or below the pI of the target protein is used. A larger difference in pH would lead to a greater net charge on the protein, multi-point adsorption and a requirement for stronger elution conditions. Normally a pH is chosen to be just sufficient to promote adsorption. This can be simply determined as shown in *Method Table 4* . If pH change is to be used as a means of protein elution, the same test outlined above can also be used to determine the most suitable elution pH (i.e. that at which protein adsorption is just prevented). A protein usually begins to dissociate from an exchanger at 0.5 of a pH unit from its pI at an ionic strength at 0.1 M NaCl (9). These simple tests give some idea of the starting and elution pH. In the resolution of multi-component mixtures some degree of trial and error is necessary since the titration curve (i.e. the net charge as a function of pH) of all the proteins present is not known. One important point should however be kept in mind. Although a protein has a net negative charge above its pI and a net positive charge below, the localization of charge clusters on a protein surface may occasionally allow its adsorption to an ion exchanger at a pH on the 'wrong' side of its pI (8). A protein with a localized area of positive charges but an overall negative charge could, therefore, by correct orientation on the ion-exchange surface, bind to a cation exchanger, and *vice versa*.

Method Table 4. Rapid metho
exchange.

1.	Add 0.5 ml wet volume
2.	Equilibrate the matrix by up at a different pH. U exchangers. Use a pH i
3.	Wash the matrix in a lo tube at the pre-selected
4.	Add a known amount of
5.	Allow to settle and assay pure or a specific assay
6.	The starting pH is that w is that which just prever

[a]An incubation time of 10 min
porous supports may require l

5.2.5 *Selection of adsorption a*

The ionic strength of the buffer
blocking of the ionogenic groups
is therefore critical in controllin
stationary and mobile phase. O
will allow protein binding is
recommended. The reasons for

(i) If the ionic strength is tc and effective elution will
(ii) Keeping the ionic strength of unwanted contaminants ble on elution minimizes
(iii) The strategy outlined ab

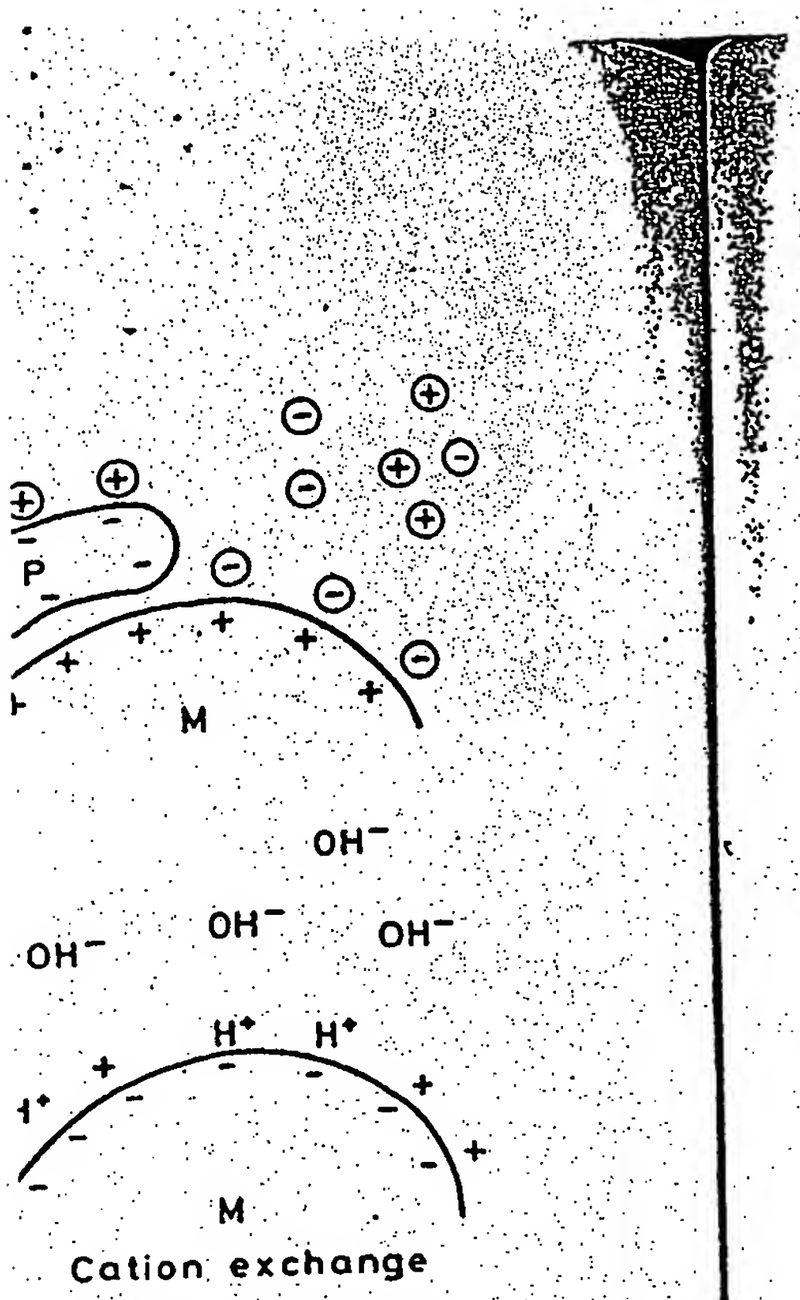
The optimum ionic strength for
in a similar fashion to the measur
is shown in *Method Table 5*. A
during adsorption and up to 0.

5.3 Procedures in ion-exchan

Having established the optimum
of a protein, certain other facto
These are the need for matrix pre

5.3.1 *Matrix pre-treatment*

Preparation of the ion exchanger
and conversion to the correct c



Cation exchange

ay cause localized pH differences in poorly

ırage protein–matrix dissociation

· below the pI of the target protein
greater net charge on the protein,
ger elution conditions. Normally a
on. This can be simply determined
ısed as a means of protein elution,
etermine the most suitable elution
evented). A protein usually begins
ıit from its pI at an ionic strength
dea of the starting and elution pH.
legree of trial and error is necessary
unction of pH) of all the proteins
owever be kept in mind. Although
d a net positive charge below, the
ay occasionally allow its adsorption
s pI (8). A protein with a localized
ıarge could, therefore, by correct
cation exchanger, and *vice versa*.

Method Table 4. Rapid method for the determination of the correct initial pH for ion exchange.

1.	Add 0.5 ml wet volume of ion exchanger to each of 10 test tubes.
2.	Equilibrate the matrix by washing 10 times in 0.5 M buffer, with each tube set up at a different pH. Use pH 4–8 for cation exchangers and 5–9 for anion exchangers. Use a pH interval of 0.5 units between each tube.
3.	Wash the matrix in a low ionic strength buffer (~20 mM) five times, in each tube at the pre-selected pH used in (2).
4.	Add a known amount of the sample protein to each tube, and mix for 10 min[a].
5.	Allow to settle and assay the supernatant for the protein using A_{280} if sufficiently pure or a specific assay if the sample contains other proteins.
6.	The starting pH is that which just allows the protein to adsorb. The eluting pH is that which just prevents adsorption.

[a]An incubation time of 10 min is based on the use of hydrogel matrices. Other less porous supports may require longer.

5.2.5 *Selection of adsorption and elution ionic strength*

The ionic strength of the buffer used in ion exchange is used to control the degree of blocking of the ionogenic groups on both protein and stationary phase. The ionic strength is therefore critical in controlling the equilibrium distribution of a protein between the stationary and mobile phase. On the adsorption step the highest ionic strength which will allow protein binding is used while on elution the lowest ionic strength is recommended. The reasons for this are threefold.

(i) If the ionic strength is too low on adsorption the protein will bind too tightly and effective elution will be made difficult.

(ii) Keeping the ionic strength as high as possible on adsorption minimizes the binding of unwanted contaminants. Conversely keeping the ionic strength as low as possible on elution minimizes elution of bound contaminants.

(iii) The strategy outlined above simplifies the elution step.

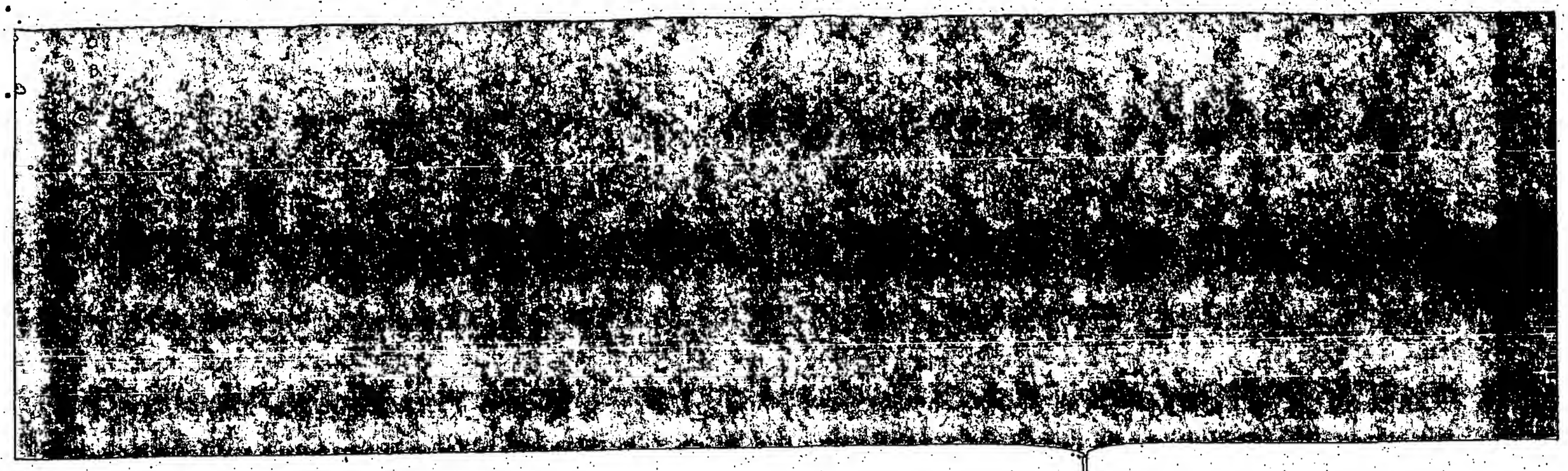
The optimum ionic strength for adsorption and elution steps can be easily determined in a similar fashion to the measurement of optimum pH outlined above. A typical method is shown in *Method Table 5*. A salt concentration of 20–50 mM NaCl is usually used during adsorption and up to 0.5 M NaCl during elution.

5.3 Procedures in ion-exchange separations

Having established the optimum adsorption and elution conditions for the purification of a protein, certain other factors must be considered before starting a purification. These are the need for matrix pre-treatment, the adsorption mode and the elution mode.

5.3.1 *Matrix pre-treatment*

Preparation of the ion exchanger for use may involve removal of fines, swelling, washing and conversion to the correct counter-ion.



Method Table 5. Rapid method for the determination of optimum ionic strength for adsorption and elution.

1.	Add 0.5 ml of ion exchanger to each of 10 tubes and equilibrate using ten 10 ml washes of 0.5 M buffer.
2.	Equilibrate each tube to a different ionic strength using 10–450 mM NaCl in 10 ml of buffer at a constant pH. Use five washes per tube.
3.	Add the sample to each tube and mix for 10 min[a].
4.	Assay the supernatant for the protein of interest.
5.	The optimum ionic strength for adsorption is that which just allows binding. The optimum ionic strength for elution is that which just prevents adsorption.

[a]An incubation time of 10 min is based on the use of hydrogel matrices. Other matrices which are less porous may require a longer time to allow for adsorption.

(i) *Fines removal.* Removal of fines has been described in Section 4.2.3. Many ion exchangers (e.g. agaroses, Sephacel, Trisacryl) are supplied relatively free of fines.

(ii) *Swelling.* Matrix swelling is necessary for supports supplied in a dry form (Sephadexes, resins, celluloses). The dry material is suspended in 10–15 bed volumes of water and left to swell for a period recommended by the manufacturer. Sephadexes require a prolonged swelling time of 2 days but this may be reduced to 2 h by incubating the slurry in a boiling water bath. Dry celluloses also require pre-cycling to disrupt hydrogen bonds and improve porosity, 0.5 M HCl and 0.5 M NaOH for anion exchangers and the reverse for cation exchangers, with each wash lasting 30 min. Precise details should be obtained from the manufacturers.

(iii) *Washing.* Certain matrices (e.g. resins) may also require washing to remove contaminants. Resins should be washed in a solvent such as methanol or acetone for 1 h to remove trapped air. They are then treated in 2 M alkali and washed in water. Cation exchangers are given an additional 2 M acid treatment and then washed again.

(iv) *Counter-ion conversion.* Ion exchangers as supplied by the manufacturer usually have a specified counter-ion associated with the ionogenic groups on the matrix. This is usually a Cl^- or OH^- counter-ion for anion exchangers and H^+ or Na^+ for cation exchangers. As mentioned earlier, counter-ions differ in their strength of attraction to ionogenic groups; if the eluent to be used contains a different counter-ion, the exchanger should be pre-treated to convert it to this form. Conditions for counter-ion conversion are shown in *Table 14*. Note that for conversion to a weaker counter-ion much larger volumes are required.

5.3.2 *Adsorption method*

Having determined the capacity and selected the optimum pH and ionic strength for both adsorption and elution steps, a choice should be made between using ion exchange in a batch or column mode.

(i) *Batch adsorption.* Batch protein fractionation is carried out in free solution and although inferior to column separations in efficiency, it is ideally suited to the initial treatment of large volumes of sample. Furthermore it does not suffer from the problems

Table 14. Conditions required for counter-

Original counter-ion	*Reqi coui*
H^+	Na^+
OH^+	Cl^-
Na^+	H^+
Cl^-	OH^-

of bed swelling and shrinkage which particularly those using dextran-base be carried out in batch mode or the and then eluted. The entire capacit adsorption step.

(ii) *Column adsorption.* As with ba used to purify a protein by adsorptic to pass through the column witho purification, no concentration of th protein is adsorbed onto the support concentration and/or fractionation.

5.3.3 *Elution method*

It is important to distinguish betwe purification.

(i) *Static ion exchange.* Here the pr completely eluted by displacement strong eluent. This method is useful i of sample.

(ii) *Dynamic ion exchange.* Here the speeds of migration through the co all the sample components migrate, i distributions between stationary and exist.

(1) Isocratic elution. Here the s the bed volume. This is be concentrated during the adso with a diameter to length r throughout the separation wi but results in large elution v

(2) Step-wise elution. This is acl pH and/or salt concentration (by the exchanger capacity ar

Table 14. Conditions required for counter-ion conversion.

Original counter-ion	*Required counter-ion*	*Procedure*
H^+	Na^+	2 vols 0.1–1 M NaOH or 2 vols 3 M NaCl
OH^+	Cl^-	2 vols 0.1–1 M HCl or 2 vols 3 M NaCl
Na^+	H^+	30 vols 0.1–1 M HCl or 30 vols 3 M NaCl
Cl^-	OH^-	30 vols 0.1–1 mM NaOH or 30 vols 3 M NaCl

of bed swelling and shrinkage which are sometimes encountered in column separations, particularly those using dextran-based matrices. Elution following batch adsorption may be carried out in batch mode or the ion exchanger slurry may be packed into a column and then eluted. The entire capacity of the ion exchanger should be used during the adsorption step.

(ii) *Column adsorption.* As with batch adsorption packed bed ion exchangers can be used to purify a protein by adsorption of contaminants. This allows the desired protein to pass through the column without binding. While this is an acceptable means of purification, no concentration of the protein results. Usually, however, the required protein is adsorbed onto the support in preference to contaminants and then eluted with concentration and/or fractionation.

5.3.3 *Elution method*

It is important to distinguish between two alternative modes of ion-exchange protein purification.

(i) *Static ion exchange.* Here the protein is initially fully adsorbed to the bed and then completely eluted by displacement into the mobile phase using a small volume of a strong eluent. This method is useful for the concentration of protein from a large volume of sample.

(ii) *Dynamic ion exchange.* Here the separation of proteins is achieved by their relative speeds of migration through the column. In contrast to the static method, therefore, all the sample components migrate, but separate depending on their relative equilibrium distributions between stationary and mobile phases. Three choices of elution conditions exist.

(1) Isocratic elution. Here the sample volume should be between 1% and 5% of the bed volume. This is because the sample is only loosely bound and not concentrated during the adsorption step. A long column is used (up to 100 cm) with a diameter to length ratio of around 1:20. The starting buffer is used throughout the separation which may give good resolution of similar proteins but results in large elution volumes.

(2) Step-wise elution. This is achieved using a sequential, discontinuous change in pH and/or salt concentration (*Figure 25*). The column volume used is determined by the exchanger capacity and the sample volume. The sample should initially

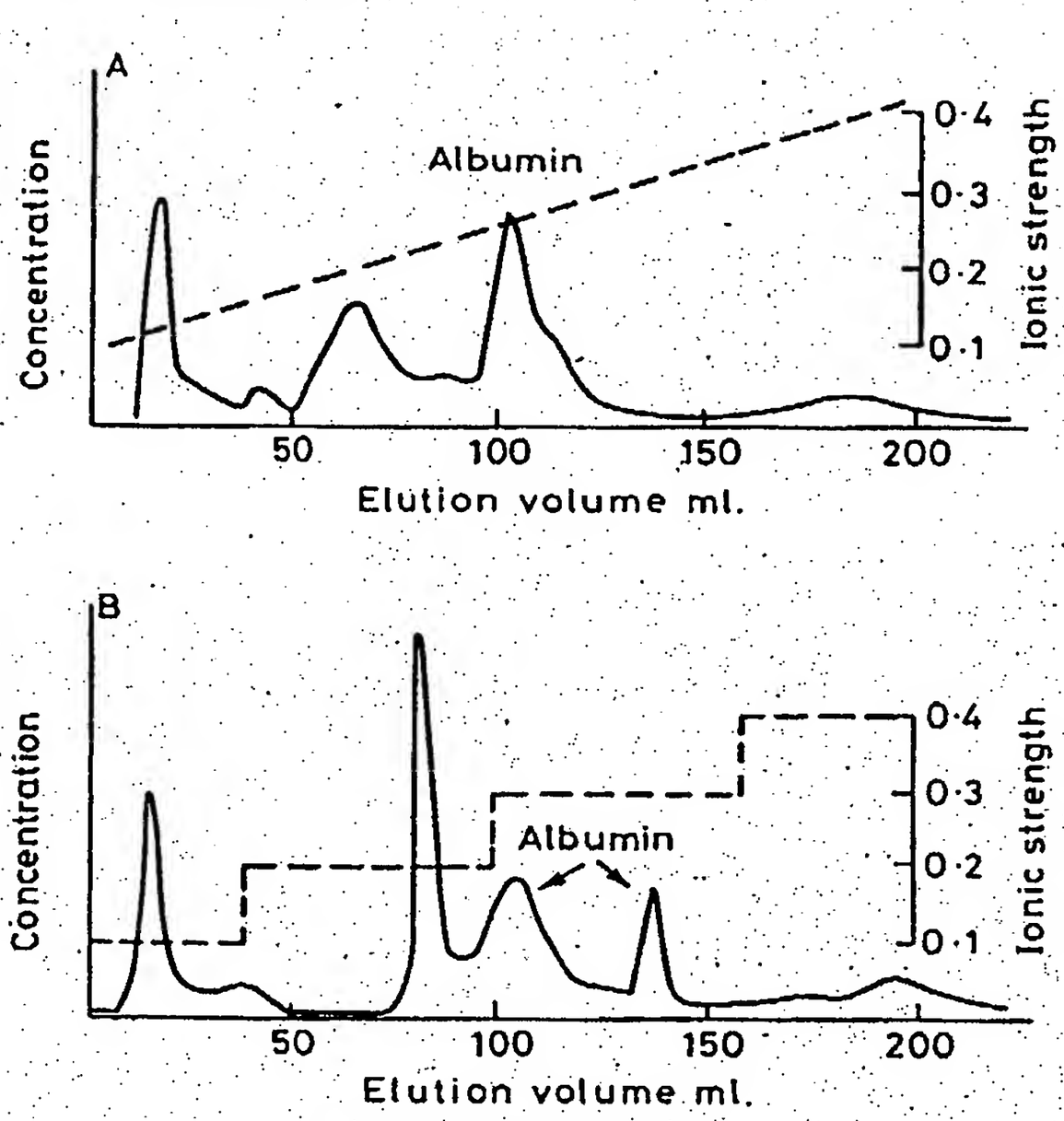


Figure 25. Continuous (A) and stepwise gradient elution of bovine serum on QAE-Sepharose A-50. In stepwise elution (B) two albumin peaks were found. This can often occur in stepwise elution when the buffer change is introduced too early. Bed dimensions, 1.5 × 26 cm; sample, 4 ml of 3% serum; eluent, 0.1 M Tris–HCl pH 6.5, 0.1–0.5 M NaCl; flow rate, 0.2 ml min^{-1}. Reproduced with permission from Pharmacia-LKB.

adsorb to 5–10% of the total bed capacity. The column length is usually shorter (20–40 cm).

(3) Gradient elution. Here the composition of the eluent (pH and/or ionic strength) is changed continuously. As in stepwise elution sample protein content should be 5–10% of the bed capacity. The column length is usually 20–40 cm with a diameter to length ratio of not more than 1:5. The volume of buffer required for elution should be determined empirically. If the gradient is too steep resolution will be lost, while too shallow a gradient will result in unnecessary dilution and long separation times. The total volume of eluent should be about five times the bed volume (9).

An increasing pH gradient may be used for cation exchangers while a decreasing pH gradient is used for anion exchangers. Continuous pH gradients are rarely used because they have poor reproducibility (9). This is due to the titration of both protein and ion exchanger ionogenic groups as the pH is altered. A changing pH gradient is also difficult to produce at constant ionic strength. Consequently elution through pH

change usually uses a stepwise m
with an increase in ionic streng

Stepwise elution uses simpler
resolution of eluted peaks may be
in elution conditions may cause t
in *Figure 25*, if a stepwise chan
peaks may be produced.

5.3.4 *Regeneration and storage*

Regeneration of ion exchangers
and the conversion of the support
and protein adsorption. Regener
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should be removed from the co

Removal of tightly bound prote
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Ion exchangers stored in a we
is particularly true for polysac
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where these preservatives cannot
ethanol) is recommended.

5.4 High performance ion-exc

A wide range of HPLC matrice
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More recently developed me
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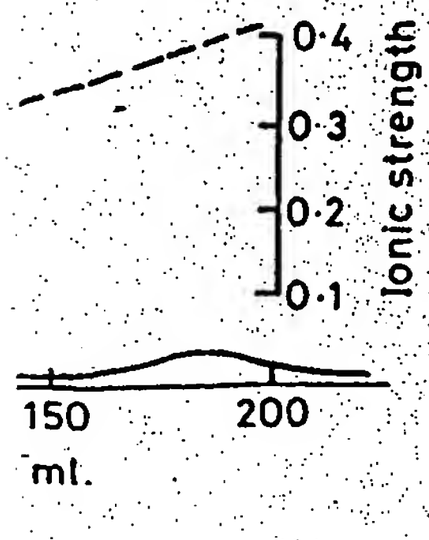

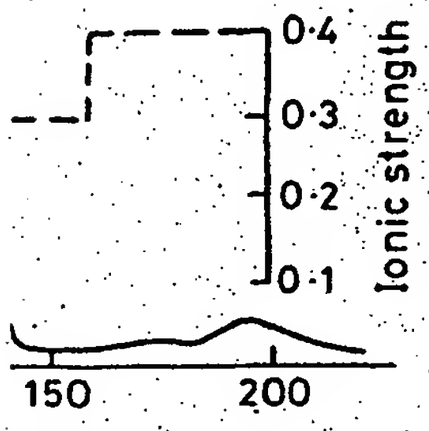

serum on QAE-Sepharose A-50. In stepwise
in stepwise elution when the buffer change
ml of 3% serum; eluent, 0.1 M Tris–HCl
ed with permission from Pharmacia-LKB.

ne column length is usually shorter

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length is usually 20–40 cm with
5. The volume of buffer required
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due to the titration of both protein
tered. A changing pH gradient is
Consequently elution through pH

change usually uses a stepwise method. Elution by a change in pH may also be combined with an increase in ionic strength.

Stepwise elution uses simpler apparatus and is usually used on a large scale. The resolution of eluted peaks may be poorer than in gradient elution since stepwise increases in elution conditions may cause the co-elution of several proteins. Furthermore, as seen in *Figure 25*, if a stepwise change in elution conditions is introduced too early false peaks may be produced.

5.3.4 *Regeneration and storage*

Regeneration of ion exchangers involves the removal of tightly bound contaminants and the conversion of the support to the required counter-ion form ready for equilibration and protein adsorption. Regeneration can be carried out in the column but for matrices such as Sephadex, where bed volume is dependent on ionic strength, the ion exchanger should be removed from the column and regenerated in free solution.

Removal of tightly bound protein is first achieved using 2 M NaCl and is then followed in some cases with an alkaline wash. The manufacturers' literature should be consulted for instructions on the precise regeneration conditions.

Ion exchangers stored in a wet state are susceptible to microbial degradation. This is particularly true for polysaccharide-based matrices. Recommended antimicrobial agents (9) for anion exchangers include 0.002% chlorohexidine (Hibitane), 0.001% phenyl mercuric salts in a mildly alkaline solution and 0.05% trichlorobutanol in a weakly acidic solution. The latter can also be used with cation exchangers, in addition to 0.005% merthiolate (ethyl mercuric thiosalicylate) in a mildly acidic solution. In applications where these preservatives cannot be used a high concentration of organic solvents (70% ethanol) is recommended.

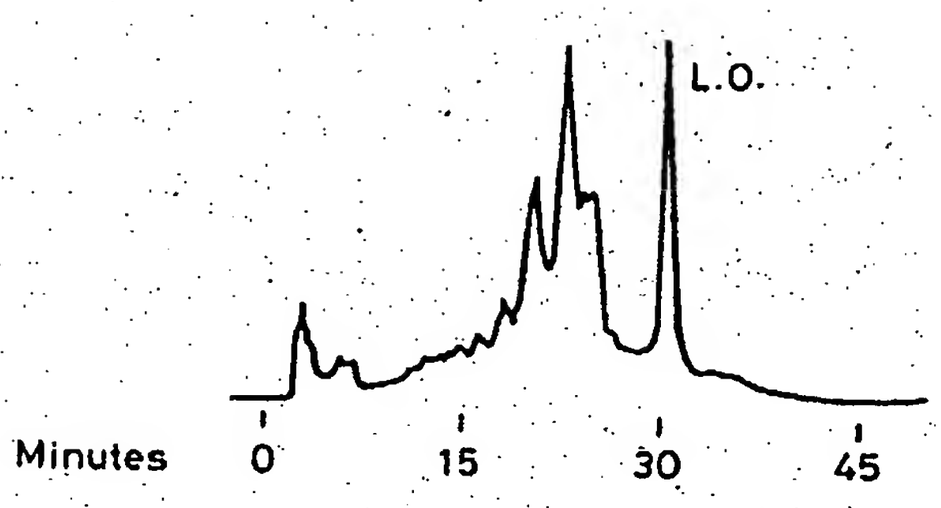
5.4 High performance ion-exchange chromatography

A wide range of HPLC matrices are now available; some have been listed in *Table 11*. Ion-exchange HPLC of proteins is usually based on wide pore silica or resin matrices. For the purification of medium molecular weight proteins (30–100 kd) pore sizes should be at least 30 nm while for larger proteins (>100 kd) pore sizes should be 500 nm or even 1000 nm in diameter.

The principles of low pressure ion-exchange can be applied to high performance separations. Analytical scale columns (typically 4.6 mm × 7–10 cm) usually separate mg quantities of protein at a time using 200 μl injection volumes, flow rates of 0.3–1.0 ml min^{-1} and a pressure of 1000 p.s.i. Typical separation times are less than 1 h. The high cost of HPLC media usually restricts its use to small volumes of sample.

More recently developed medium pressure packings include Accell (Waters) and Monobeads (Pharmacia). Accell packing is a 37–55 μm silica-based material with large pores. It is used at flow rates of up to 200 ml cm^{-2} h^{-1} to provide protein separations in 30 min to 2 h either in pre-packed or laboratory-packed glass columns. Accell is available with anion (quaternary methylamine) and cation (carboxymethyl) functionalities; the typical capacity is 30–40 mg ml^{-1} (BSA). The monobead packings (Mono Q and Mono S) are based on a hydrophilic polymer resin of size 10 ± 0.5 μm. The matrix is highly porous ($m^{ex} > 10^7$), is typically used at flow rate of 150–200 ml

Separation based on structure



Column: Bio-Gel TSK DEAE-5PW (Cl- form) 75 x 7.5 mm

Sample: Commercial preparation of lipoxidase, 1 mg in 0.1 ml

Eluant: pH 9.0
Solvent A: 0.02M ethanolamine HCl
Solvent B: 0.5M NaCl in 0.02M ethanolamine-HCl

Linear Gradient: 0% to 100% B over 60 min.

Figure 26. Purification of commercial lipoxidase using ion exchange HPLC. Reproduced with permission from Bio-Rad.

$cm^{-2}\ h^{-1}$ and can withstand back pressures of up to 750 p.s.i. Separation times are usually between 20 min and 1 h. The capacity of the ion exchangers is quoted as 25 mg ml^{-1}.

For larger scale and lower cost purifications Pharmacia recommend using Sepharose Fast Flow which can only be used at back pressures of up to 25 p.s.i.; the separation times are therefore significantly longer but still an improvement on conventional low pressure packings.

5.5. Separation using chromatofocusing

Chromatofocusing, first described and experimentally verified by Sluyterman and co-workers (29,30) can be considered as an extension of isoelectric focusing and ion-exchange chromatography. In isoelectric focusing, proteins are separated by electrophoresis in a pH gradient in a matrix produced by a current. In ion exchange, proteins are bound to the column at an initial pH and may then be eluted by a changing pH gradient. This is produced by mixing a 'limit' buffer (of different pH) with the initial buffer in a mixing chamber and then pumping this through the column (*Figure 27*). The change in mobile phase pH partitions the protein into the mobile phase where it is eluted from the column. In chromatofocusing the pH gradient is produced inside the column by mixing an anion exchange matrix, pre-adjusted to one pH, with a buffer at a second lower pH (*Figure 27*). The protein sample is applied to the column in the

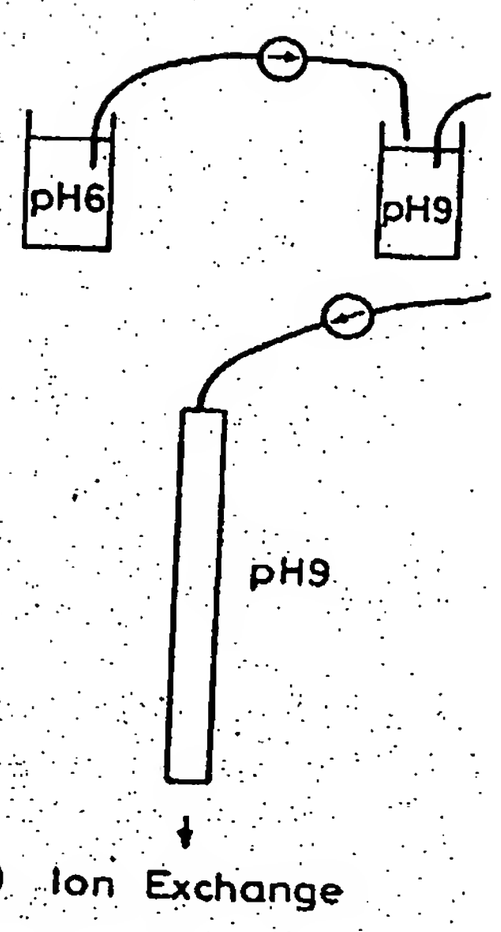
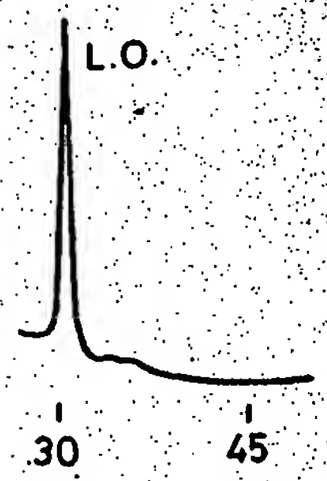


Figure 27. Chromatofocusing differs from ion properties so that a pH gradient is produced

Figure 28. A protein of pI 9 applied to a col column in the pH 8 elution buffer until it reac

elution buffer or the starting buffer. A a column pre-equilibrated at pH 10 will and experience a rise in pH due to the t reaches a pH just above its pI it will bec



IEAE-5PW

preparation of
ng in 0.1ml

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B over 60 min.

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ion exchangers is quoted as 25 mg

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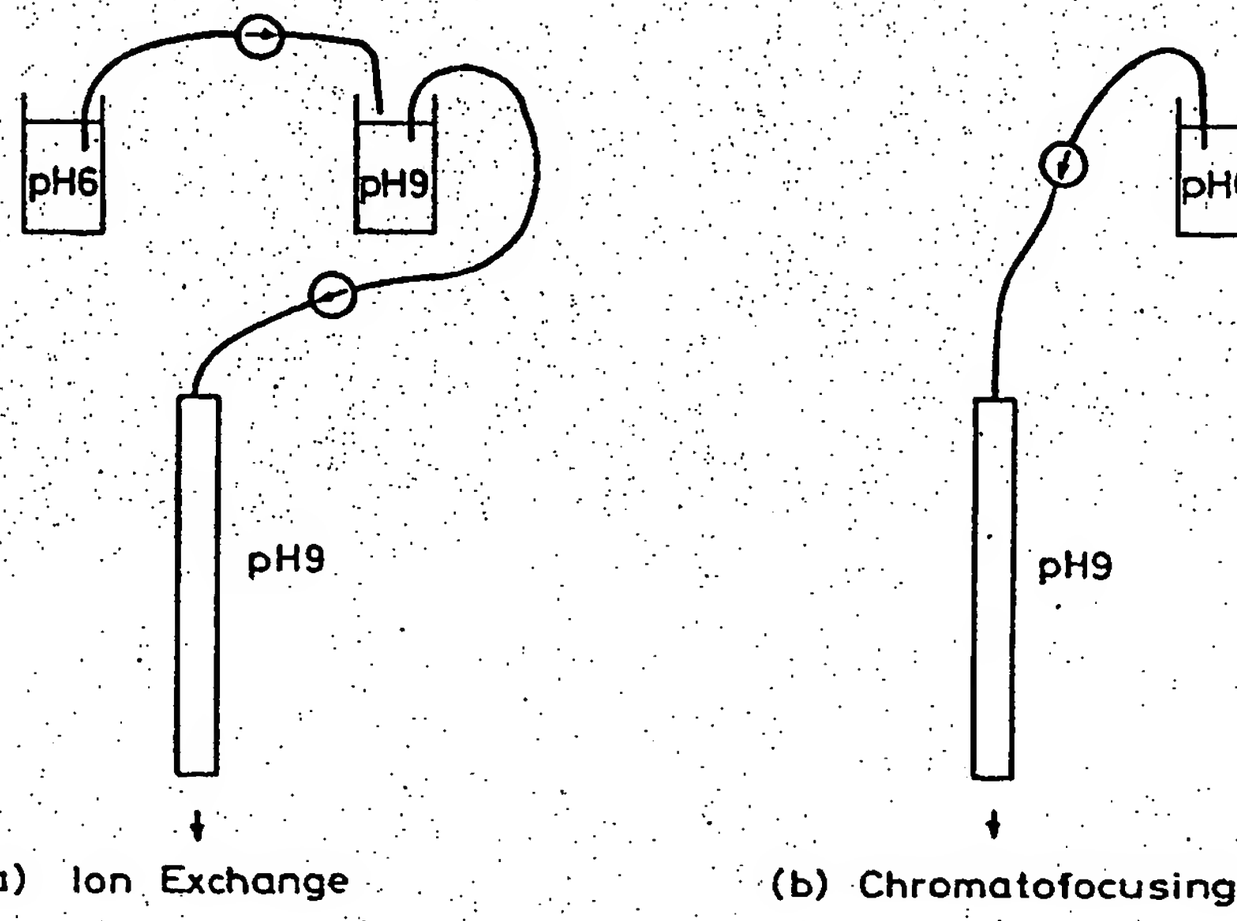

Figure 27. Chromatofocusing differs from ion exchange in that the anion exchange matrix has distinct buffering properties so that a pH gradient is produced on the column rather than outside the column.

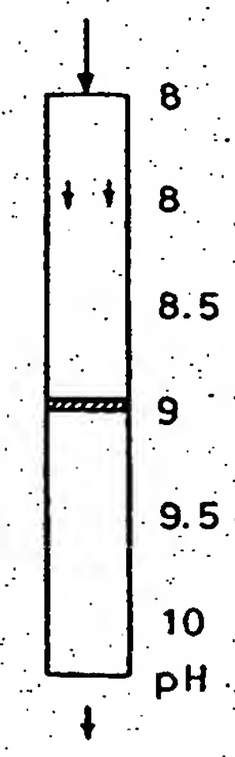

Figure 28. A protein of pI 9 applied to a column pre-equilibrated to pH 10 will move rapidly down the column in the pH 8 elution buffer until it reaches its isoelectric pH where it will bind to the exchanger.

elution buffer or the starting buffer. A protein of pI 9 in a pH 8 buffer pumped onto a column pre-equilibrated at pH 10 will therefore move with the buffer down the column and experience a rise in pH due to the buffering action of the column. When the protein reaches a pH just above its pI it will become negatively charged and bind to the positively-

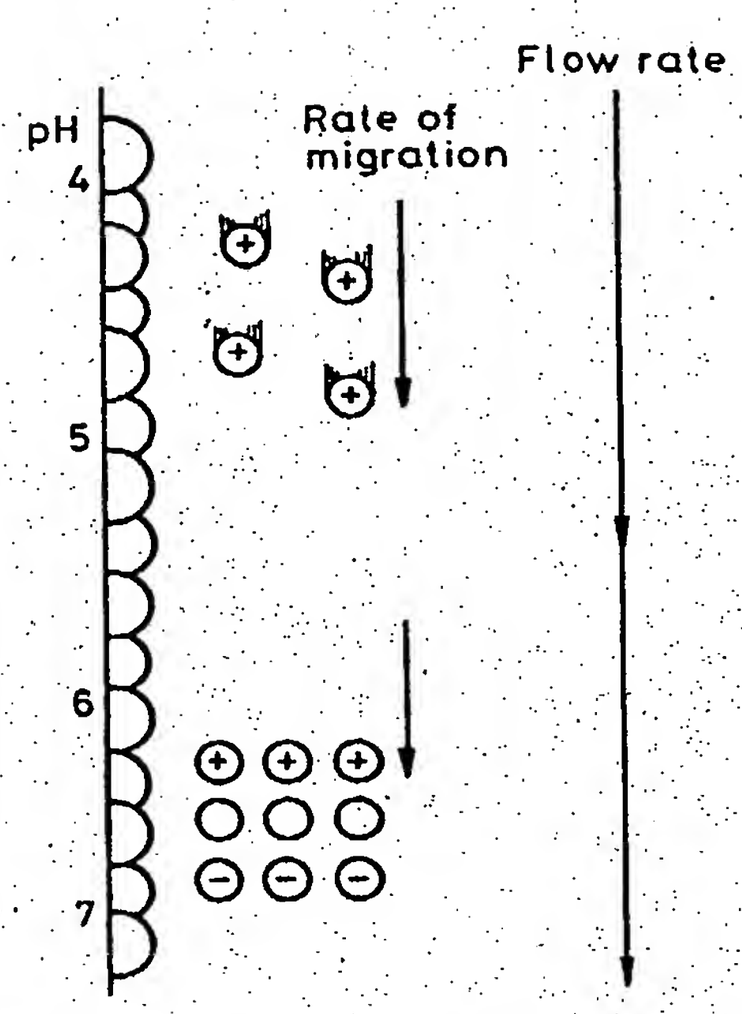

Figure 29. The focusing effect of chromatofocusing. Proteins migrate through the column slower than the flow rate of buffer. Proteins at a pH close to their pI migrate slower than proteins higher up the column at a lower pH, allowing them to catch the leading band up. Reproduced with kind permission from Pharmacia-LKB.

charged column matrix. (*Figure 28*). As elution buffer flows through the column the bound protein will experience a lowering of the pH until it becomes positively charged again and moves with the buffer down the column to reach a second point where the pH is again just above its pI and adsorption occurs. This sequence of events is repeated many times until the protein is eluted from the column, at its isoelectric pH. The protein is never subjected to a pH more extreme than its pI value; the separation is therefore mild.

A further important feature of chromatofocusing is the focusing effect which operates during the separation. A protein applied to the column will travel with the elution buffer to a point where it is negatively charged and then binds to the exchanger, until more elution buffer flows down the column, lowering the pH still further. Any additional protein applied in a second aliquot to the column can therefore catch up with the initial bound protein (*Figure 29*). This serves as a peak sharpening effect in chromatofocusing, allowing resolution of complex mixtures of proteins, providing they differ in their pI's.

Due to the focusing effect of chromatofocusing, proteins can be applied to the column in large volumes without impairing the resolution. The maximum volume in which a protein can be dissolved in order to emerge as a single band is termed the distribution volume. This is governed by the pI of the protein and the equilibration pH. If a protein's pI is close to the initial column pH the protein will be carried rapidly down the column in the elution buffer to be eluted in the void column; the distribution volume is then near zero. Conversely if the pI is close to the elution buffer pH then the protein distribution volume will be very large.

Table 15. Chromatofocusing media and b

pH range	
11–8	
9–6	
7–4	
12–2	

[a]Mono P is a 10-μm matrix for use in the

Table 16. Starting buffers for use in chrc

Upper pH unit
9
8
7
6
5

5.5.1 *Experimental conditions an*

The initial studies on the feasibility (30,31) used DEAE–Sepharose a are important in the selection of l

(i) The column matrix must al groups and the buffer. Polye was subsequently chosen ir

(ii) The ionic strength of the buf The buffering capacity shoul the near-linear pH gradient was best achieved using ampl buffers (e.g. triethylamine)

Although chromatofocusing usi (31,32), the resolution of comple designed to combine a high capacity reproducible pH gradients. To thi (*Table 15*) and ampholyte buffers a large number of protein separatio using both low pressure and FPL

The choice of exchanger and oper used in elution. This in turn is det pI of the protein should be appro gradient (33).

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(i) If the pI of the protein is no If the protein pI is above 7 i from 9 to 6 should be used

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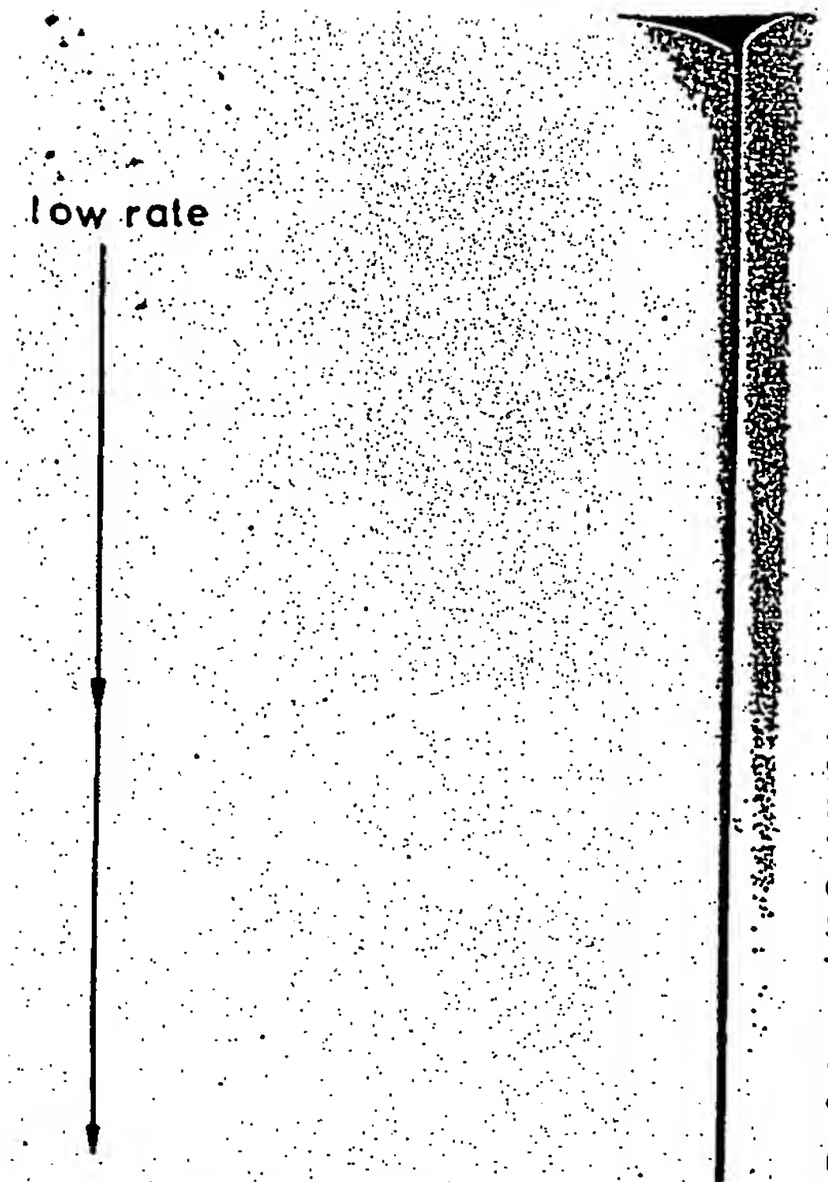

grate through the column slower than the
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fer flows through the column the
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carried rapidly down the column
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ion buffer pH then the protein

Table 15. Chromatofocusing media and buffers.

pH range	*Exchanger*	*Elution buffer*
11–8	PBE 118	Pharmalyte 8–10.5
9–6	PBE 94	Polybuffer 96
7–4	PBE 94	Polybuffer 74
12–2	Mono P[a]	Any of the above

[a]Mono P is a 10-μm matrix for use in the Pharmacia-LKB FPLC system.

Table 16. Starting buffers for use in chromatofocusing.

Upper pH unit	*Buffer*	*Required pH*
9	Ethanolamine–HCl	9.4
8	Tris–HCl	8.3
7	Imidazole–HCl	7.4
6	Histidine–HCl	6.2
5	Piperazine–HCl	5.5

5.5.1 *Experimental conditions and chromatofocusing*

The initial studies on the feasibility of chromatofocusing by Sluyterman and co-workers (30,31) used DEAE–Sepharose and acetate buffer. The conclusions from this work are important in the selection of both column media and suitable buffers.

(i) The column matrix must allow rapid pH equilibration between ion exchange groups and the buffer. Polyethyleneimine attached to epoxide-activated Sepharose was subsequently chosen in preference to DEAE–Sepharose.

(ii) The ionic strength of the buffer should be kept relatively constant during elution. The buffering capacity should be constant over the entire pH range so as to provide the near-linear pH gradient necessary for obtaining optimum resolution. This was best achieved using ampholyte buffers during elution although normal cationic buffers (e.g. triethylamine) are used for pre-equilibration.

Although chromatofocusing using conventional ion exchange materials is possible (31,32), the resolution of complex protein mixtures is best achieved using matrices designed to combine a high capacity with rapid equilibration and buffers providing linear, reproducible pH gradients. To this end Pharmacia-LKB market a range of matrices (*Table 15*) and ampholyte buffers (*Table 16*) for use in chromatofocusing (33), and a large number of protein separations have been achieved using these materials (34–41) using both low pressure and FPLC.

The choice of exchanger and operating buffers depends on the range of the pH gradient used in elution. This in turn is determined by the pI of the protein to be purified: the pI of the protein should be approximately midway between the pH extremes of the gradient (33).

A procedure for the use of chromatofocusing is described in *Method Table 6*. In addition, the following points should be noted.

(i) If the pI of the protein is not known a pH gradient from 7 to 4 is used initially. If the protein pI is above 7 it will not bind to the exchanger and a pH gradient from 9 to 6 should be used.

Method Table 6. Procedure for the separation of proteins using chromatofocusing.

1.	Determine the protein pI and select the appropriate exchanger, starting and elution buffer from *Tables 15* and *16*.
2.	Mix the exchanger with half its bed volume of starting buffer, pre-adjusted to −0.3 pH units above the upper limit of the gradient. Degas and pour into the column. 10 ml of exchanger per 100 mg of protein is normally adequate. Use a long narrow column e.g. 1 × 40 cm.
3.	Equilibrate the column with 10−15 bed volumes of starting buffer at a flow rate of 100 ml cm^{-2} h^{-1}.
4.	Dilute the elution buffer 1−10 with water; adjust to the lower pH limit of the gradient. Equilibrate the sample with starting or elution buffer. Its volume may be large but is usually not over half the column void volume. Its ionic strength should be <0.05 M.
5.	Apply the sample to the column at 40 ml cm^{-2} h^{-1} and then begin elution. A gradient volume of 10 column volumes is normally sufficient.

(ii) The amount of exchanger required for a separation will depend on the purity of the sample, its volume and its protein content. For samples containing up to 200 mg of protein per pH unit of the gradient, bed volumes of 20−30 ml are recommended. The volume of sample is not critical if loading onto the column is finished prior to elution of the protein of interest.

(iii) The pH of the start buffer is usually adjusted to 0.4 of a pH unit above the required pH to compensate for fluctuations in pH on application of the elution buffer. This is due to a release of protons from the exchanger during the early stages of elution.

(iv) The stronger the elution buffer the shorter the pH gradient and the lower the resolution. A pH gradient of over 3 units is not recommended. For the highest resolution, a narrow pH gradient should be used.

(v) Proteins should be monitored at the outlet using UV absorption at 280 nm, since elution buffers absorb at 240 nm and below.

Chromatofocusing can provide very good resolution of complex mixtures of proteins (*Figure 30*) provided they do not form a continuous distribution of isoelectric points, in which case adjacent protein peaks will overlap with loss of resolution. The presence of non-protein charged material (i.e. nucleic and fatty acids) may also reduce the efficiency of separation. Proteins can be eluted within a pH window of 0.04−0.05 of a unit giving sharp well resolved peaks provided the gradient is not run too fast, in which case loss of resolution will occur (33). Chromatofocusing has also been used with samples containing 7 M urea, 1% (v/v) Triton X-100, 1% (v/v) Tween 80, 5% (v/v) DMSO and 50% (v/v) ethylene glycol, but additives causing an increase in ionic strength should be avoided. The feasibility of using chromatofocusing for protein purification depends on the acceptability of both the cost of the ampholyte buffers required for elution and their presence in the protein sample after purification.

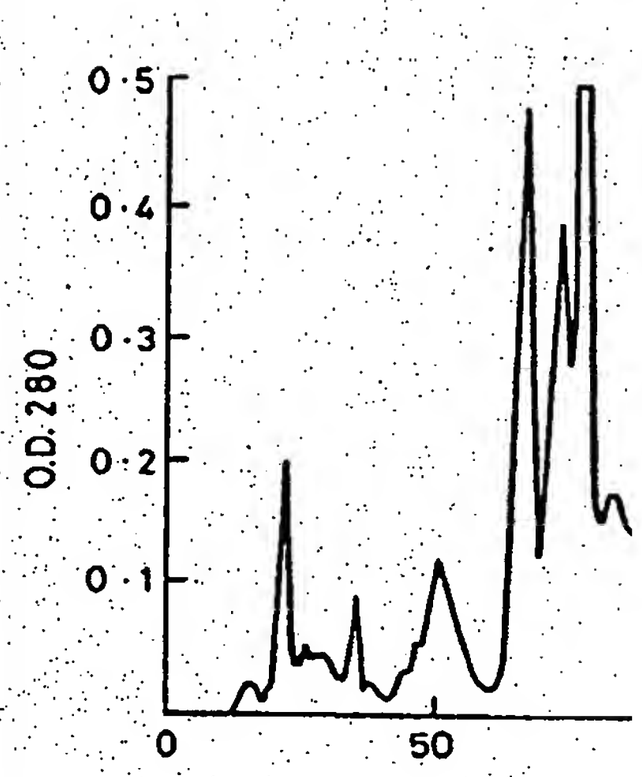

Figure 30. Fractionation of elk muscle pı
5 ml of muscle extract. Start buffer 0.02
Flow rate 20 cm^2 h^{-1}. Reproduced with

5.5.2 *Exchanger regeneration an*

Exchangers can be regenerated w
Strongly bound proteins can be re
re-equilibrated to a more neutral
Exchangers should be stored in 2
(Pharmacia-LKB) but should be

6. PURIFICATION BASED ON

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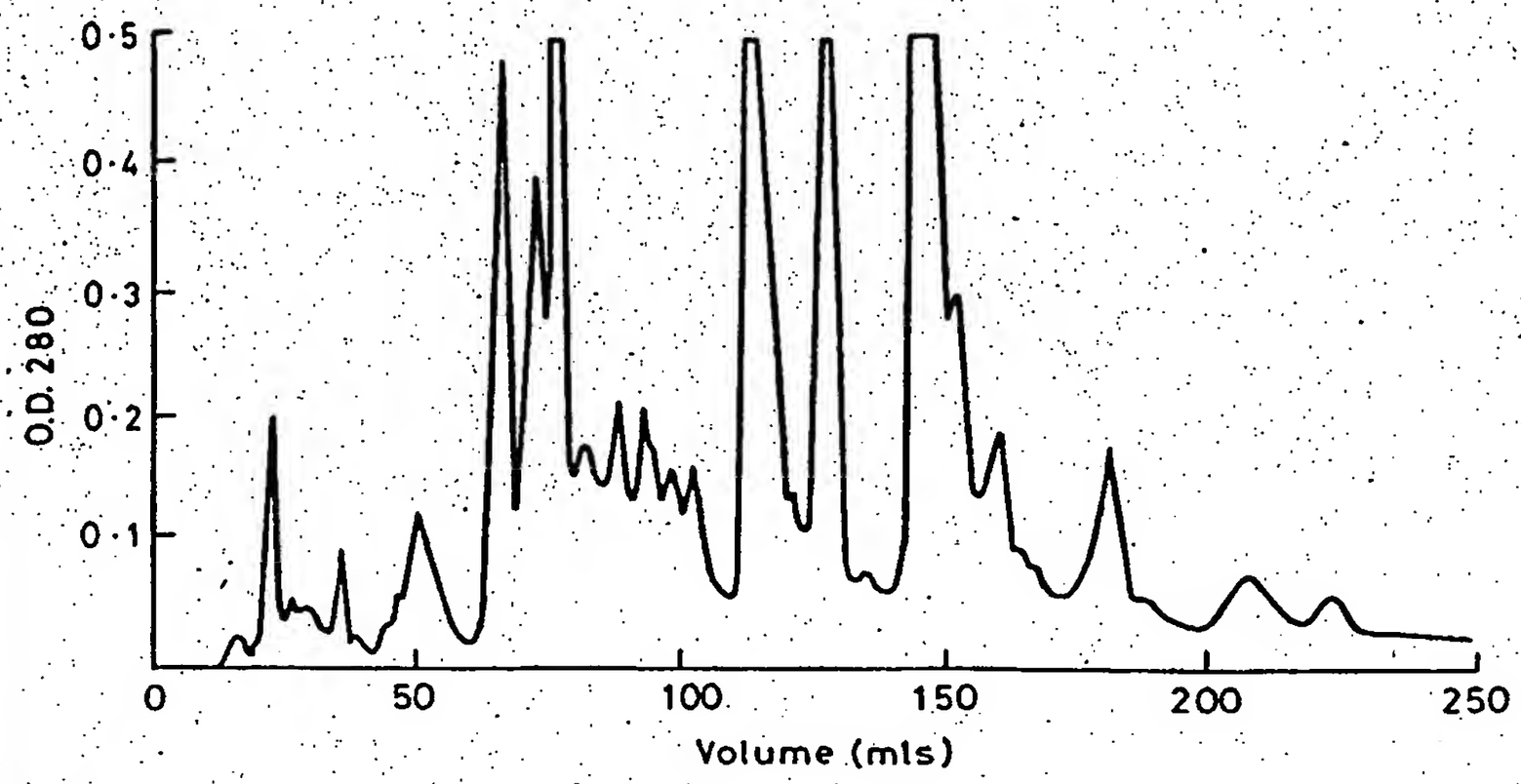

Figure 30. Fractionation of elk muscle proteins by chromatofocusing. Column 10 mm × 40 cm. Sample 5 ml of muscle extract. Start buffer 0.025 M ethanolamine–HCl, pH 9.4. Elution polybuffer 96, pH 6. Flow rate 20 cm^2 h^{-1}. Reproduced with kind permission of Pharmacia-LKB.

5.5.2 *Exchanger regeneration and storage*

Exchangers can be regenerated without removal from the column using 1 M NaCl. Strongly bound proteins can be removed using 0.1 M HCl provided the exchanger is re-equilibrated to a more neutral pH as soon as possible.

Exchangers should be stored in 24% ethanol, while elution buffers are supplied sterile (Pharmacia-LKB) but should be stored cold in the dark.

6. PURIFICATION BASED ON HYDROPHOBICITY

Reverse-phase chromatography (RPC) is the separation of solutes on the basis of their distribution between a polar mobile phase and an organic phase which is fixed to a matrix. Along with the contrasting technique of normal phase chromatography (using a polar stationary phase and organic mobile phase) these techniques were originally termed partition chromatography, since the two separation phases were essentially liquid, one being immobilized to a stationary support. The slow leakage of the immobilized liquid phase meant, however, that steps were subsequently taken chemically to modify the support, thereby fixing the surface film in place. RPC commonly uses aliphatic chains of up to 18 carbon atoms (C_{18}) chemically bonded to silica. The mobile phase is polar, consisting mostly of water with the addition of polar solvents such as methanol, propanol, ethanol and acetonitrile to promote displacement of the solute from the non-ionic stationary phase into the mobile phase. The combination of a highly hydrophobic stationary phase and the solvents used to promote elution may cause protein denaturation. This is largely due to the disruption of protein tertiary structure caused by interaction with the high density of alkyl groups attached to the silica packing (3). In order to

minimize the risk of denaturation, less densely clustered alkyl groups of a milder hydrophobic nature (e.g. C_8) are required. Under these conditions, proteins can usually be eluted using a decreasing salt gradient. This has been termed hydrophobic interaction chromatography (HIC). The hydrophobicity of a support therefore determines whether it is used in reverse-phase or hydrophobic mode.

The occurrence of non-ionic interactions in protein separation was originally reported as an interference effect resulting from the aliphatic spacer arms used in affinity chromatography (42). Subsequent work on the coupling of alkyl and aryl-amines to agarose gels (43–46) using the cyanogen bromide technique (47) provided amphiphilic *N*-substituted isoureas with both ionic and hydrophobic groups. The combination of both effects led to a complex mode of adsorption involving both non-ionic and electrostatic interactions (45,46). Electrically neutral adsorbents based on alkyl chains attached to agarose were consequently synthesized (48) allowing hydrophobic interaction without electrostatic interaction.

6.1 Theory of hydrophobic interaction chromatography

The simplistic model of protein tertiary structure envisages an essentially hydrophilic outer shell surrounding a hydrophobic core. However, surface hydrophobicity does occur due to the presence at the surface of the side chains of non-polar amino acids such as alanine, methionine, tryptophan and phenylalanine (49). It is likely that surface hydrophobicity not only helps to stabilize protein conformation but forms the basis of specific interactions connected with the biological function of the protein. These may include antigen–antibody, hormone–receptor and enzyme–substrate type interactions and are therefore of significant biological importance (50). The surface hydrophobic amino acids are usually arranged in patches, interspersed with more hydrophilic domains. The number, size and distribution of these non-ionic regions is a characteristic of each protein and can therefore be used as a basis for their separation (51).

A protein molecule in solution holds a film of water in an ordered structure at its surface which must be removed from non-ionic domains before hydrophobic interaction can occur as outlined in *Figure 31*. Removal of bound water molecules from the protein surface into the less ordered bulk solution results in an increase in entropy ($\Delta S > 0$). The overall free energy change (ΔG) for the interaction of two non-ionic groups in the adsorption step is related to entropy and enthalpy as follows:

$$\Delta G = \Delta H - T\Delta S \qquad (1)$$

Since the enthalpy change (ΔH) in the interaction is small, the free energy change of the process is negative and therefore proceeds spontaneously (50–52). The likelihood of a non-ionic interaction is further increased if the excluded water molecules are trapped in the bulk solution by the hydration of salt ions. Consequently it is convenient to use HIC following ammonium sulphate precipitation since the ions which are most effective at salting proteins out of solution are generally those which create the most structuring in water. The hydrophobic interaction between protein surface non-ionic groups at high salt concentration forms the basis of protein precipitation using ammonium sulphate (see Chapter 3). In HIC, however, the interacting non-ionic group (e.g. octyl/phenyl) is provided by a hydrophobic functionality attached to an inert matrix such as agarose.

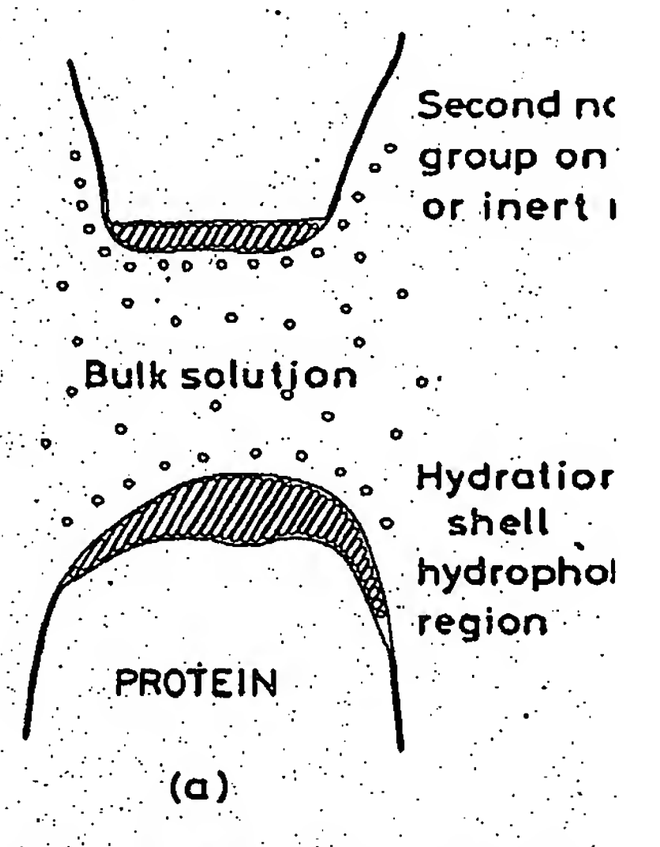

Figure 31. Model of non-ionic exchange i
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excludes the bound water in a spontaneous

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PO_4^{2-}, SO_4^{2-}, CH_3CO

NH_4^+, Rb^+, K^+, I

Incr

Figure 32. The influence of ions on hydro

Nevertheless, the adsorption step o
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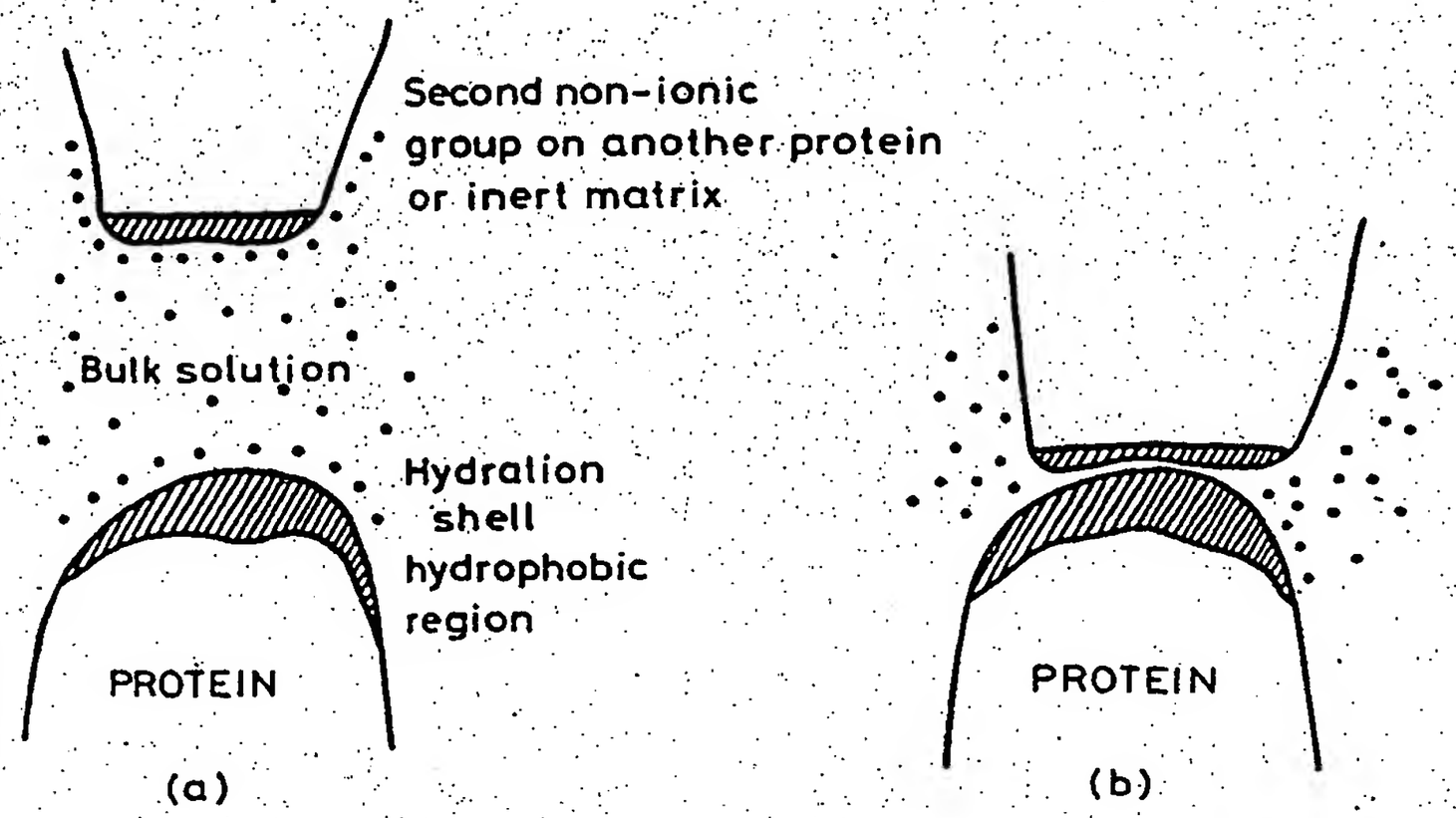

Figure 31. Model of non-ionic exchange interaction. (a) Protein in solution with a shell of water around its hydrophobic region. (b) Exclusion of water into bulk solution upon approach of another non-ionic group excludes the bound water in a spontaneous process driven by the gain in entropy.

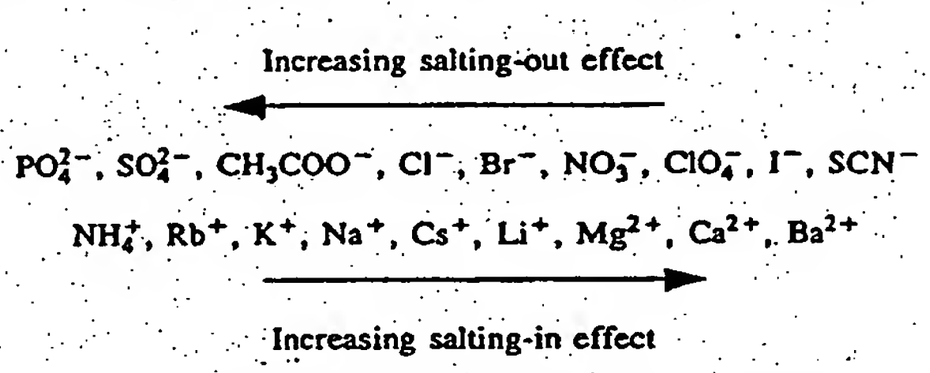

Figure 32. The influence of ions on hydrophobic interactions. (The Hofmeister series.)

Nevertheless, the adsorption step often requires the presence of salting-out ions such as sodium chloride or ammonium sulphate. The influence of certain ions on protein structure was first reported by Hofmeister (53). Salting-out ions decrease the availability of water molecules in solution, increase the surface tension and enhance hydrophobic interaction. In contrast, salting-in or chaotropic ions such as sodium thiocyanate prevent non-ionic interaction by ordering the structure of water (15). Their use in elution from hydrophobic matrices is, however, usually avoided since they may denature proteins. Chaotropic and salting-out ions are shown in *Figure 32*.

Temperature also influences the level of hydrophobic interaction. As equation (1) states, the free energy of the process becomes more negative with increasing temperature (up to ~60°C where the additional stability provided by electrostatic and Van der Waal's forces disappears). The reduced strength of HIC interaction at lower temperatures is not, however, usually significant enough to be used as a means of protein elution.

Protein and adsorbent charge may also have an important effect on the degree of hydrophobic adsorption. A protein with no net charge (i.e. at its pI) will have maximum

Table 17. List of some commonly used hydrophobic adsorbents.

Manufacturer/ Supplier	*Packing*	*Comments*
Low/medium pressure packings		
Miles	Butyl, hexyl, octyl agarose	Capacity—10 mg protein ml^{-1}
Pharmacia	Octyl, Phenyl Sepharose	Capacity—20 mg ml^{-1}
	Phenyl Superose	Capacity—10 mg ml^{-1}
	Alkyl Superose	Particle size 10 μm For use in FPLC
Serva	Daltosil Octyl	Silica based
	Phenyl, Aminohexyl	Pore size 50 nm
	Aminopropyl	Particle size 0.1–0.2 mm
	Aminophenyl	
Waters	Protein-Pak HIC	10 μm particle size
	Phenyl 5PW	Used in glass columns
HPLC packings		
Alltech	Synchropack Propyl	6.5 μm silica
Anachem	Anagel TSK/HIC	
Beckman	Spherogel TSK-Phenyl	
	Spherogel CAA-HIC	5 μm particles 30 nm pores
	[a]Ultrapore C3	5 μm silica
Bio-Rad	Bio-Gel Tsk Phenyl	10 μm particles, 100 nm pore
	[a]Hi-Pore RP	Silica C4
Brownlee	Aquapore RP300	Butyl or Phenyl on 7 μm silica
Perkin Elmer	TSK-Phenyl	
[a]Phase-Sep	Spherisorb C_1, C_3 Octyl, Phenyl	30 nm pores 5, 10 μm particles
[a]Shandon	Hypersil—Butyl, Octyl	5, 10 μm silica 30 nm pores
[a]Vydac	C_4, diphenyl	
Waters	μBondapak-Phenyl	

[a]Normally used in reverse-phase chromatography.

hydrophobicity. At a pH where the protein and adsorbent have similar charges repulsion may occur, resulting in reduced adsorption.

Other conditions which reduce adsorption include polarity lowering agents such as water miscible solvents (e.g. ethanol and ethylene glycol) and detergents. Their use in elution is usually considered a last resort when other milder conditions such as reduced conductivity do not promote protein recovery.

6.2 Experimental conditions for

6.2.1 *Adsorption*

The most commonly used non-ion
phenyl functionalities. Many others
in *Table 17*. The stationary phase
a decrease in ionic strength will enc
desorption. The choice of the hyc
determining the ease of subsequen

Phenyl Sepharose, for example,
Extremely hydrophobic proteins (e
Octyl Sepharose and require very s
therefore be used. In contrast, milc
Sepharose and will therefore requi

The solution ionic strength will al
its degree of adsorption. Mildly hy
ionic strength is increased to just
hydrophobic proteins such as globu
at low salt concentration (20–40%
ionic strength should be adjusted w
required protein to enhance non-ioni
equilibrated. The sample is then app
The capacities of hydrophobic matı
10–100 mg ml^{-1} of adsorbent) (E

6.2.2 *Elution conditions*

A variety of conditions can be usec
a potentially powerful method for th
include the following.

(i) Reducing the ionic strength
(ii) Increasing the pH: most pro
hydrophilic under mildly al
(iii) Reducing the temperature
earlier. Generally however,
on its own as an effective r
(iv) Displacement methods: addi
for the ligand or makes the
(1) *Aliphatic alcohols.* (
reduce the polarity of
(2) *Aliphatic amines.* (e.
the solution polarity c
tein or matrix hydrop
(3) *Detergents.* Non-ionic
displace bound protei

Ionic detergents (e.g. sodium do

nts.

	Comments
	Capacity—10 mg protein ml^{-1}
	Capacity—20 mg ml^{-1}
	Capacity—10 mg ml^{-1}
	Particle size 10 μm
	For use in FPLC
	Silica based
	Pore size 50 nm
	Particle size 0.1–0.2 mm
	10 μm particle size
	Used in glass columns
	6.5 μm silica
	5 μm particles 30 nm pores
	5 μm silica
	10 μm particles, 100 nm pore
	Silica C4
	Butyl or Phenyl on 7 μm silica
	30 nm pores 5, 10 μm particles
	5, 10 μm silica 30 nm pores

rbent have similar charges repulsion

le polarity lowering agents such as
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6.2 Experimental conditions for low pressure HIC

6.2.1 *Adsorption*

The most commonly used non-ionic adsorbents are based on agarose using octyl or phenyl functionalities. Many others exist and some are listed, along with HPLC packings in *Table 17*. The stationary phase should be charge-free or subsequent elution using a decrease in ionic strength will encourage electrostatic interaction and prevent effective desorption. The choice of the hydrophobic ligand is of considerable importance in determining the ease of subsequent protein elution.

Phenyl Sepharose, for example, is less hydrophobic than Octyl Sepharose (54). Extremely hydrophobic proteins (e.g. membrane proteins) may adsorb too strongly to Octyl Sepharose and require very strong eluting conditions. Phenyl Sepharose should therefore be used. In contrast, mildly hydrophobic proteins may not adsorb to Phenyl Sepharose and will therefore require use of more hydrophobic matrices.

The solution ionic strength will also control the protein hydrophobicity and therefore its degree of adsorption. Mildly hydrophobic proteins may not be adsorbed until the ionic strength is increased to just below that required for precipitation. The more hydrophobic proteins such as globulins will associate with other non-ionic groups even at low salt concentration (20–40% w/v ammonium sulphate). Prior to HIC, the sample ionic strength should be adjusted with salt and buffered preferably near the pI of the required protein to enhance non-ionic adsorption. The packed column should be similarly equilibrated. The sample is then applied to the matrix and washed with the same buffer. The capacities of hydrophobic matrices are usually as high as for ion exchangers (i.e. 10–100 mg ml^{-1} of adsorbent) (8).

6.2.2 *Elution conditions*

A variety of conditions can be used for elution from hydrophobic matrices, providing a potentially powerful method for the resolution of complex mixtures of proteins. These include the following.

(i) Reducing the ionic strength using either isocratic or gradient elution.
(ii) Increasing the pH: most proteins gain a net negative charge and become more hydrophilic under mildly alkaline conditions.
(iii) Reducing the temperature should theoretically promote elution as described earlier. Generally however, the temperature effect is too small for it to be used on its own as an effective means of elution.
(iv) Displacement methods: addition of a component which has a stronger attraction for the ligand or makes the protein more hydrophilic.
  (1) *Aliphatic alcohols.* (e.g. propanol, butanol and ethylene glycol). These reduce the polarity of the solution and disrupt hydrophobic interaction.
  (2) *Aliphatic amines.* (e.g. butylamine). These have the effect of reducing the solution polarity causing desorption. The amines may bind to the protein or matrix hydrophobic groups.
  (3) *Detergents.* Non-ionic detergents (e.g. Tween 20, Triton X-100) probably displace bound proteins by binding to both protein and column packing.

Ionic detergents (e.g. sodium dodecyl sulphate) are more easily removed from the

Separation based on structure

Method Table 7. Procedure for protein isolation by HIC using sequential elution with reduced ionic strength and a displacer.

1. Suspend the matrix in half its bed volume of 0.8 M ammonium sulphate in 0.025 M potassium phosphate buffer pH 7, degas and pour as a slurry into the column.
2. Wash the column with three column volumes of the starting buffer.
3. Apply the sample in the same buffer.
4. Elute with 3.5 bed volumes of a decreasing linear gradient of ammonium sulphate (0.8–0 M) in 0.025 M potassium phosphate buffer pH 7.0.
5. Elute with four bed volumes of a linear increasing gradient of 0–80% (v/v) ethylene glycol.

Method Table 8. Hydrophobic interaction chromatography involving the use of a displacer during adsorption.

Matrix : Phenyl Sepharose CL-4B (50 ml)
Column : K-26/40 (2.9 × 9.4 cm)
Application : Purification of natural human fibroblast interferon (IFN).

1. Remove fines and degas matrix, pack into column.
2. Rinse with 0.5 litres of 40% (v/v) ethylene glycol in 0.02 M sodium phosphate buffer pH 7 + 0.15 M NaCl (PBS).
3. Apply 1 litre of preparation containing 40% ethylene glycol in PBS at a flow rate of 10.5 ml cm^{-2} h^{-1}.
4. Wash with 0.3 litres of 40% (v/v) ethylene glycol in PBS.
5. Recover IFN with 0.3 litre of 75% (v/v) ethylene glycol in PBS. Dilute immediately with 0.25 litre PBS.

column after protein desorption. They tend, however,to cause protein denaturation. Non-ionic detergents are milder and can be used at levels of 1–3% without causing loss of activity (8).

Frequently, a gradient of decreasing ionic strength is used followed by a gradient of an increasing concentration of a displacer to ensure desorption as given in *Method Table 7*. If the desired protein is very hydrophobic, displacers (55) may be used in the initial adsorption conditions to prevent adsorption of other less hydrophobic contaminants (*Method Table 8*). Protein desorption may also be achieved using one-step elution (56) at reduced ionic strength. An example is given in *Method Table 9*. Both reducing ionic strength and increasing displacer gradients (57) may however be applied at the same time (*Method Table 10*).

Detergents may also be used in tandem in applications where strong hydrophobic adsorption has occurred. In the example shown in *Method Table 11*, 1.5% Tween 80 is used to remove cholate from the column during cytochrome *c* oxidase purification (58). The enzyme itself is then eluted using 1% (w/v) Triton X-100 which acts as a stronger non-ionic detergent.

Method Table 9. One-step elutio

Matrix : Phenyl Sepharose CL-4
Column : 2.6 × 26 cm
Application : Human interleukin

1. Remove fines, degas and p
2. Equilibrate with 1 M ammo pH 6.8.
3. Apply sample.
4. Wash with 450 ml of the s
5. Elute with 800 ml of 0.2 M pH 6.8.

NB In this example it was necessar human IL1.

Method Table 10. Gradient elu displacer.

Matrix : Octyl Sepharose CL-4B
Column : K16/20 (bed volume 3
Application: β-amylase purificatic

1. Remove fines and degas m
2. Wash with 0.01 M sodium sulphate.
3. Apply sample (40 ml in th
4. Wash with 85 ml of the sa
5. Elute with a gradient of dec and increasing ethylene gl

The elution conditions necessary empirically. A decreasing ionic str always be used initially. Failure by using stronger elution condition matrix.

6.2.3 *Matrix regeneration and st*

Prior to re-use, any tightly bound urea, and the support washed in elution should be removed with a by two bed volumes of butan-1-c of distilled water (54). The bed is t the gels are suspended in 0.02% (at 2–8°C.

HIC using sequential elution with

of 0.8 M ammonium sulphate in
gas and pour as a slurry into the

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ple is given in *Method Table 9*.
gradients (57) may however be

tions where strong hydrophobic
ethod Table 11, 1.5% Tween 80
tochrome *c* oxidase purification
v) Triton X-100 which acts as a

Method Table 9. One-step elution using reduced ionic strength.

Matrix : Phenyl Sepharose CL-4B
Column : 2.6 × 26 cm
Application : Human interleukin 1 (IL1) purification

1. Remove fines, degas and pack column.
2. Equilibrate with 1 M ammonium sulphate in 10 mM potassium phosphate buffer pH 6.8.
3. Apply sample.
4. Wash with 450 ml of the same buffer.
5. Elute with 800 ml of 0.2 M ammonium sulphate and 10 mM phosphate buffer pH 6.8.

NB In this example it was necessary to avoid the use of ethylene glycol since it denatures human IL1.

Method Table 10. Gradient elution using reduced ionic strength and increasing displacer.

Matrix : Octyl Sepharose CL-4B
Column : K16/20 (bed volume 30 ml)
Application: β-amylase purification

1. Remove fines and degas matrix, pack into column.
2. Wash with 0.01 M sodium phosphate buffer pH 6.8 + 25% (w/v) ammonium sulphate.
3. Apply sample (40 ml in the same buffer). Flow rate = 25 ml h^{-1}.
4. Wash with 85 ml of the same buffer.
5. Elute with a gradient of decreasing ammonium sulphate concentration (25–0%) and increasing ethylene glycol (0–5%) simultaneously.

The elution conditions necessary for optimum protein separation must be determined empirically. A decreasing ionic strength gradient (down to just water), should, however, always be used initially. Failure to elute the required protein can then be overcome by using stronger elution conditions (e.g. ethylene glycol) or by using a less hydrophobic matrix.

6.2.3 *Matrix regeneration and storage*

Prior to re-use, any tightly bound protein should be removed from the matrix with 6 M urea, and the support washed in starting buffer. Any detergents used during protein elution should be removed with an increasing ethanol gradient (up to 95%) followed by two bed volumes of butan-1-ol, one bed volume of ethanol and one bed volume of distilled water (54). The bed is then washed with starting buffer. For prolonged storage the gels are suspended in 0.02% (v/v) merthiolate or 20–25% (v/v) ethanol and stored at 2–8°C.

Method Table 11. Use of two detergents in hydrophobic interaction chromatography.

Matrix : Octyl Sepharose CL-4B
Application : Cytochrome *c* oxidase purification

1. Remove fines, degas matrix and pack into column.
2. Equilibrate with Tris–cholate buffer pH 8 + 15% (w/v) ammonium sulphate, 1 mM EDTA.
3. Apply sample to column in same buffer at 4 ml min^{-1}.
4. Wash with 10% (w/v) cholate/50 mM Tris–sulphate (pH 8) + 1 mM EDTA.
5. Elute cholate with 1.5% (v/v) Tween-80 in 50 mM Tris–sulphate.
6. Elute cytochrome *c* oxidase with 1% (v/v) Triton X-100 in 50 mM Tris–sulphate.

6.3 High performance HIC

The influence of support hydrophobicity on the risk of protein denaturation has already been mentioned and should be kept in mind in the selection of HPLC packings. Certain matrices are now recommended for HIC and these have been listed in *Table 17*.

In HPHIC, separations can be achieved in 30–60 min using flow rates of 0.5–1 ml min^{-1} (*Figure 33*). Slower flow rates (60–90 ml cm^{-2} h^{-1}; <0.4 ml min^{-1}) are however preferred. In the selection of column packings wide pore supports (>30 nm) are preferable for improved resolution; with pore sizes of 6–10 nm only surface adsorption of protein will occur (3).

Finally, care should be taken to wash the HPLC system out with water after use to remove all traces of salts and minimize corrosion of metal pump components.

Pharmacia-LKB also produce hydrophobic matrices for use in their FPLC system. The packings are based on 10 μm agarose particles (Superose) for use at flow rates of 150 ml cm^{-2} h^{-1} at a pressure of up to 400 p.s.i. The matrix has a capacity of 10 mg ml^{-1} allowing purification of up to 80 mg of protein at a time.

6.4 Advantages and disadvantages of HIC

Hydrophobic interaction chromatography provides a powerful additional means of separation which is applicable to the purification of most proteins. It is ideal for use immediately after salt precipitation where the ionic strength of the sample will enhance hydrophobic interaction. In purification where the required protein is eluted in a gradient of decreasing ionic strength, it can be followed by ion exchange with little need for buffer change.

The diversity of potential eluting conditions can enable the resolution of even complex mixtures of proteins which would be difficult to separate by other chromatographic techniques. Predicting the best conditions for separation is, however, difficult and an element of trial and error is involved in process optimization. The effectiveness of HIC is generally reduced by the presence of hydrophobic contaminants in the feed. An additional problem encountered in the use of HIC is the strong conditions which are frequently required to elute proteins from hydrophobic matrices. This may involve the

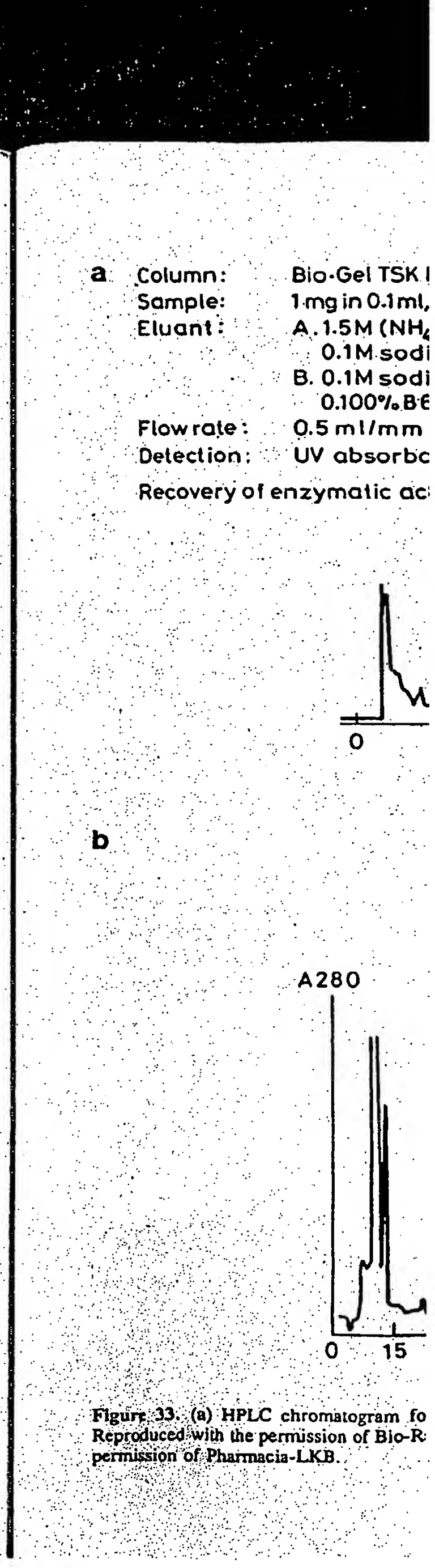

Figure 33. (a) HPLC chromatogram fo
Reproduced with the permission of Bio-R
permission of Pharmacia-LKB.

bic interaction chromatography.

lumn.
15% (w/v) ammonium sulphate,

ml min^{-1}.
ılphate (pH 8) + 1 mM EDTA.
0 mM Tris–sulphate.
X-100 in 50 mM Tris–sulphate.

protein denaturation has already
ction of HPLC packings. Certain
ıave been listed in *Table 17*.
ıin using flow rates of 0.5–1 ml
m^{-2} h^{-1}; <0.4 ml min^{-1}) are
ıs wide pore supports (>30 nm)
ıizes of 6–10 nm only surface

stem out with water after use to
f metal pump components.
s for use in their FPLC system.
(Superose) for use at flow rates
i. The matrix has a capacity of
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ength of the sample will enhance
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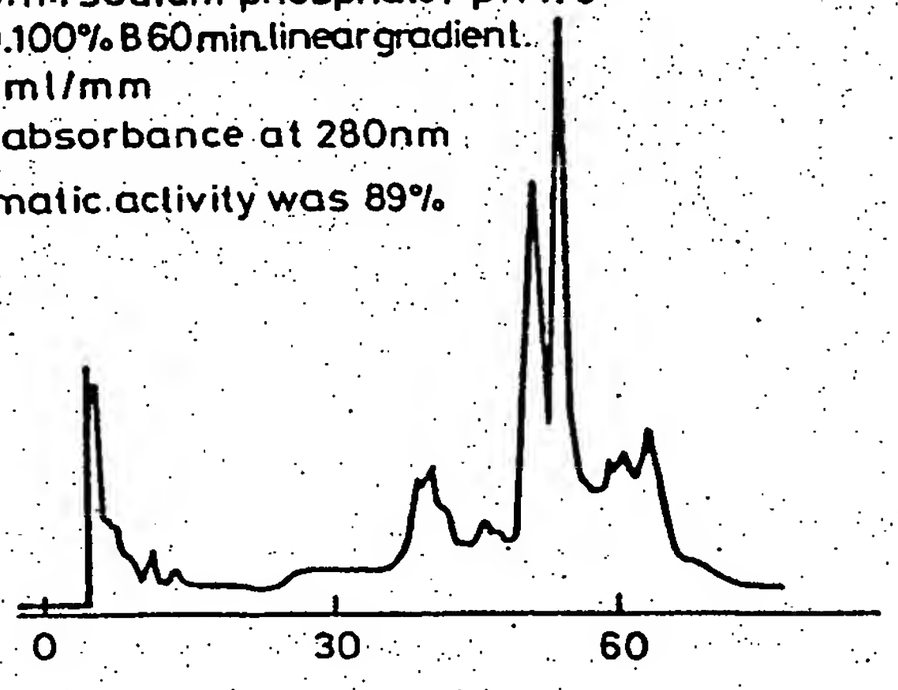
ıle the resolution of even complex
ıarate by other chromatographic
ion is, however, difficult and an
ization. The effectiveness of HIC
ic contaminants in the feed. An
the strong conditions which are
c matrices. This may involve the

a

Column: Bio-Gel TSK Phenyl-5PW, 75x7.5 mm I.D.
Sample: 1 mg in 0.1 ml, commercial lipoxidase
Eluant: A. 1.5 M $(NH_4)_2SO_4$
0.1 M sodium phosphate, pH 7.0
B. 0.1 M sodium phosphate, pH 7.0
0.100% B 60 min linear gradient.
Flow rate: 0.5 ml/mm
Detection: UV absorbance at 280nm
Recovery of enzymatic activity was 89%



b

Milk protein purification
Sample: Fermented milk whey
Buffer A, 1.2,200 µl
Column: Alkyl-Superose HR 5.5
Flow rate: 0.5 ml/min
Buffer A: 0.1 M phosphate, pH 7.0, 2.0 M $(NH_4)_2SO_4$
B: 0.1 M phosphate, pH 7.0
Gradient: 0% B for 5 min. 0–100% B in 30 min.

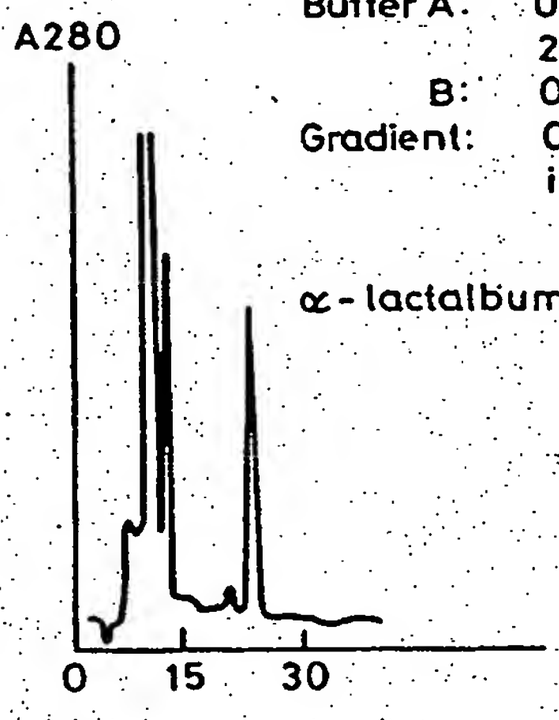


Figure 33. (a) HPLC chromatogram for the purification of lipoxidase using hydrophobic interaction. Reproduced with the permission of Bio-Rad. (b) FPLC purification of milk proteins. Reproduced with the permission of Pharmacia-LKB.

Table 18. List of some commonly used reverse-phase packings.

Manufacturer	*Packing name*	*Comments*
Alltech	Synchrom RP	6.5 μm silica, 30 nm pores C_1, C_4, C_8, C_{18}
Amicon	Matrex	10–20 μm silica C_8, C_{18}
Anachem	Dynamax	12 μm silica C_8
Anachem	Bulo-Series PEP RP1	5 μm silica C_8
Beckman	Ultrapore C_3	5 μm silica 30 nm pores
Bio-Rad	Hi-Pore RP	Silica C_4 or C_{18}
DuPont	Zorbax PolyF	20 μm
DuPont	Zorbax Bio-Series	4 μm silica C_8
Perkin Elmer	HCODs C_{18}	5, 10 μm silica
Pharmacia-LKB	Pro RPC	5, 15 μm silica
Phase-Sep	Spherisorb	C_1–C_{18} Octyl, Phenyl
Shandon	Hypersil WP300	Butyl, Octyl, 5, 10 μm silica
Shandon	ODS, MOS Hypersil	C_8, C_{18} silica
Vydac	Vydac C_4, C_{18}	5 and 10 μm silica
Waters	μ Bondapak	C_{18}, CN, Phenyl
	Delta-Pak	15 μm silica C_4, C_{18}
Whatman	Protesil 300	Octyl silica 30 nm pores

Table 19. Eleutropic series of solvents for use in reverse-phase chromatography: stronger solvents have a greater eluting power for proteins on reverse-phase columns.

Increasing polarity	↑	Water
		Methanol
		Acetonitrile
		Ethanol
		Tetrahydrofuran
		N-Propanol
	↓	Decreasing polarity

use of non-ionic detergents or ethylene glycol which can increase the likelihood of protein denaturation. The use of strong elution conditions may also be required when the reduction in ionic strength during elution encourages electrostatic interactions from charged groups on the adsorbent matrix. The use of HPLC for HIC must involve the regular washing out of salts so as to prevent corrosion.

6.5 Reverse-phase chromatography (RPC)

Reverse-phase techniques have traditionally been applied to the analysis of low molecular weight compounds using HPLC. It is characterized by the use of silica derivatized with alkyl functionalities (typically C_2–C_{18} alkyl chains) and an aqueous mobile phase containing an organic solvent such as methanol, propanol, acetonitrile and ethanol. Some commercially available reverse-phase packings are listed in *Table 18*.

In addition to ligand density the length of the alkyl chain is proportional to the packing hydrophobicity. Consequently C_{18} packings bind proteins more tightly and are more likely to cause denaturation than C_8 or C_4 packings. Packings should always be chosen in which unreacted silanol groups are 'end-capped' using blocking agents such as trimethyl-chlorosilane. This minimizes interference adsorption of protein onto the

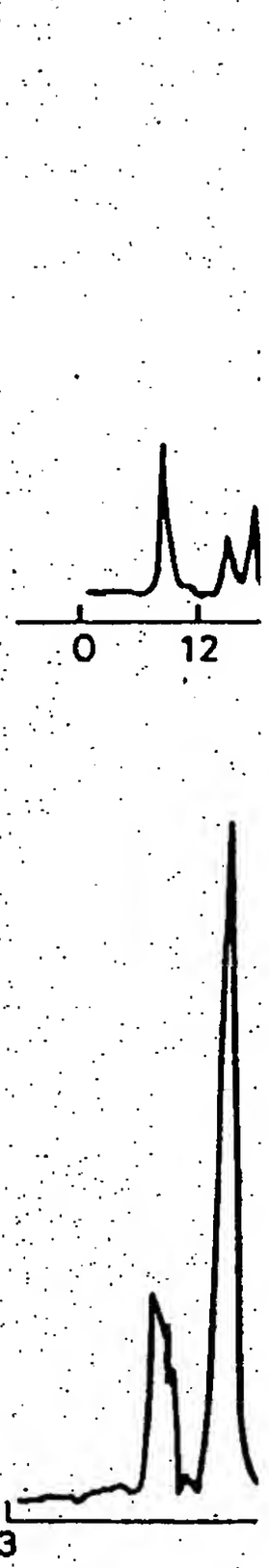


Figure 34. HPLC separations of proteins u from Whatman. (a) Separation of human min^{-1}, gradient 0–100% 1-propanol. (b) ml min^{-1}.

unreacted silanol groups which m
Furthermore, for protein purifica
in order to maximize capacity and
essential, should be checked before

Comments
6.5 μm silica, 30 nm pores C_1, C_4, C_8, C_{18}
10–20 μm silica C_8, C_{18}
12 μm silica C_8
5 μm silica C_8
5 μm silica 30 nm pores
Silica C_4 or C_{18}
20 μm
4 μm silica C_8
5, 10 μm silica
5, 15 μm silica
C_1–C_{18} Octyl, Phenyl
Butyl, Octyl, 5, 10 μm silica
C_8, C_{18} silica
5 and 10 μm silica
C_{18}, CN, Phenyl
15 μm silica C_4, C_{18}
Octyl silica 30 nm pores

: chromatography: stronger solvents
imns.

Decreasing polarity

un increase the likelihood of protein
y also be required when the reduc-
rostatic interactions from charged
for HIC must involve the regular

ied to the analysis of low molecular
y the use of silica derivatized with
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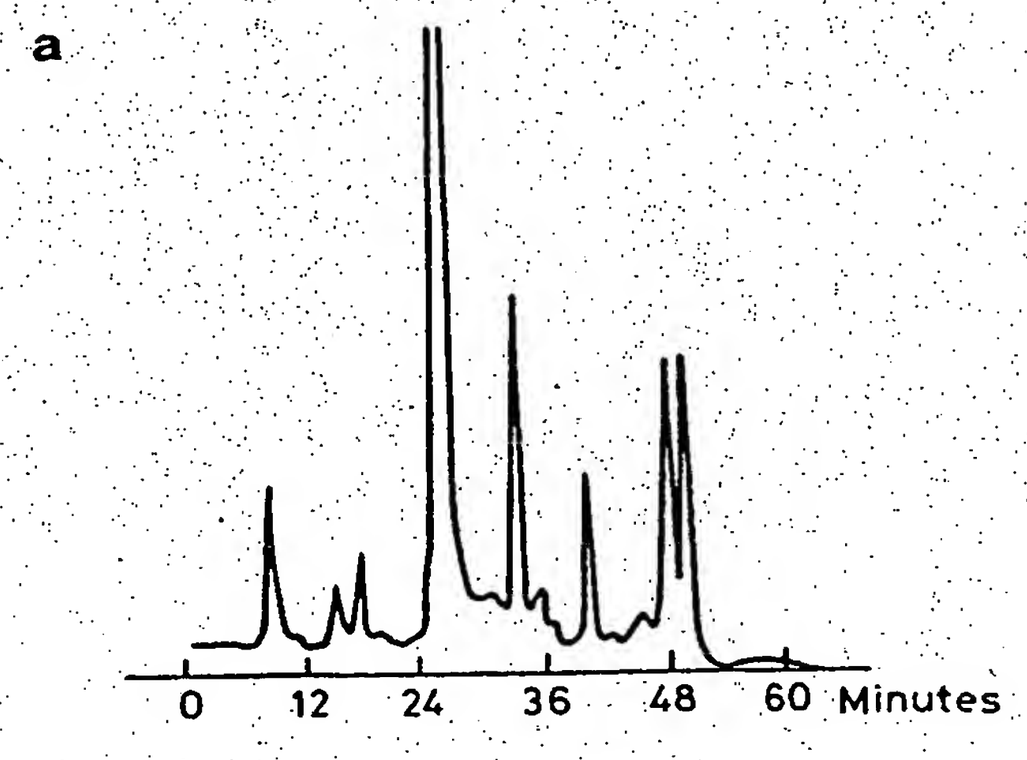

Figure 34. HPLC separations of proteins using reverse-phase chromatography. Reproduced with permission from Whatman. (a) Separation of human serum proteins using Protesil 300 Diphenyl. Flow rate: 0.5 ml min^{-1}, gradient 0–100% 1-propanol. (b) Separation of protein standards using Protesil 300. Flow rate 0.5 ml min^{-1}.

unreacted silanol groups which may cause loss of resolution and poor reproducibility. Furthermore, for protein purification, wide pore matrices (~30 nm) should be used in order to maximize capacity and resolution. Preservation of biological activity, when essential, should be checked before proceeding further with any selected column packing.

Separation based on structure

The mobile phase may be buffered (1 M strength) using sodium acetate or pyridinium formate at pH 4–6 to minimize protein adsorption to unreacted silanol groups; this can be visualized as a narrowing of peak width. Protein retention is controlled using alteration of the polarity of the mobile phase by the addition of organic solvents. The most commonly used solvents may be arranged in an eleutropic series (*Table 19*). Inclusion of 'stronger' solvents such as acetonitrile and tetrahydrofuran in the mobile phase will give a greater reduction in retention time than the 'weaker' solvents such as ethanol and methanol. The optimum solvent composition depends on the packing used and the sample composition and is therefore a matter of trial and error. HPLC grade low-UV absorbance solvents should always be used and caution should be exercised in their handling (particularly tetrahydrofuran and acetonitrile). Examples of reverse-phase HPLC separations are shown in *Figure 34*.

Elution is achieved using gradient or isocratic conditions. Gradient conditions may be preferred to reduce the separation time or improve the resolution of certain areas of chromatogram. Usually the strength of the solvent is increased during gradient elution, to 40–80% (v/v) propanol or acetonitrile in water. Recently the use of the RPC has been extended to the Pharmacia FPLC system. The matrices are based on highly porous silica (5 or 1.5 μm) for use in glass columns at pressures up to 1500 p.s.i. The matrix recommended for protein purification is Pro RPC; like all silica matrices, its pH stability is restricted to between 2 and 8. Pro RPC is a wide pore matrix (300 nm) with a mixture of functional groups (C_1 or C_2 mixed with C_8). Separations typically take 30–90 min at a flow rate of 60 ml cm^{-2} h^{-1}.

Protein retention may also be controlled by the addition of counter-ions. These are usually aliphatic molecules with a charged group which associate with the charged groups of a protein, increasing its hydrophobicity. Commonly used counter-ions (ion-pairing agents) include heptane sulphonate, tetra-*n*-butylammonium hydroxide, trifluoroacetic acid (TFA) or triethylammonium phosphate/acetate. TFA is volatile and therefore easy to remove after purification.

Another method for altering the retention of proteins in RPHPLC is to change the operating pH such that relative hydrophobicity of the protein components is altered.

6.6 Advantages and disadvantages of reverse-phase chromatography

In the purification of peptides or proteins where retention of the native state is not essential, RPC provides a high resolution technique which, using HPLC, can often separate proteins not resolvable using other chromatographic methods. In the purification of peptide hormones for example, it therefore represents a powerful technique. Its drawbacks are in the frequent use of toxic solvents such as acetonitrile and in its limited application to protein purification where denaturation is unacceptable. Note that silica based matrices will dissolve above pH 7.5.

7. METAL CHELATE CHROMATOGRAPHY

Metal chelate chromatography (59), developed by Porath and co-workers in 1975, is a refinement of ligand exchange chromatography (60) for the purification of high molecular weight and conformationally unstable biopolymers. In ligand exchange chromatography sorption occurs between the sorbate and matrix via coordination bonds

of a complex-forming ion. This l
complexation to metal cations retai
In metal chelate chromatography
surface histidine, cysteine and trypt
and Zn^{2+}.

The metal ion must be held in
to ensure efficient adsorption. This
to epichlorohydrin-activated agaros
which would then complex with met
activated agarose may be achiev
However, iminodiacetate-substitut
Iminodiacetate has also been coupl
a macroporous polyhydroxymethyl
high performance metal chelate
(Pharmacia-LKB) has been used to
isoinhibitors (64), mammalian inte
and albumin (68).

7.1 Theory of metal chelate chr

The complexation of the transition
cysteine thiol groups (59) is pH-de
strongest level of adsorption. Under
provides a stronger but less selectiv
with histidine and cysteine residu
magnesium and calcium, although t
less than for copper and zinc (69).
metal cations is also suspected (65

Metal chelate chromatography the
such as ion exchange and sorption
does not take place directly but oc
complex once formed must be suf
irrespective of salt and non-electr
proteins according to the surface d
and therefore involves a highly sel

Method Table 12. Method for the

1.	Add 125 g of epoxy-activat
	containing 20 g of the disod
2.	Shake gently for 24 h at 60
3.	Cool and wash the gel on a
	(i) 0.1 M sodium carbon
	(ii) 0.01 M sodium acetat
	(iii) water.

ısing sodium acetate or pyridinium
to unreacted silanol groups; this
ɔtein retention is controlled using
addition of organic solvents. The
an eleutropic series (*Table 19*).
and tetrahydrofuran in the mobile
: than the 'weaker' solvents such
ıposition depends on the packing
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: used and caution should be exer-
n and acetonitrile). Examples of
ıre 34.
ıditions. Gradient conditions may
ve the resolution of certain areas
s increased during gradient elution,
Recently the use of the RPC has
atrices are based on highly porous
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: all silica matrices, its pH stability
re matrix (300 nm) with a mixture
rations typically take 30–90 min

dition of counter-ions. These are
ı associate with the charged groups
ly used counter-ions (ion-pairing
ıonium hydroxide, trifluoroacetic
TFA is volatile and therefore easy

ins in RPHPLC is to change the
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on of the native state is not essen-
using HPLC, can often separate
methods. In the purification of
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h as acetonitrile and in its limited
is unacceptable. Note that silica

ɔrath and co-workers in 1975, is
50) for the purification of high
opolymers. In ligand exchange
nd matrix via coordination bonds

of a complex-forming ion. This has traditionally been accomplished using sorbate complexation to metal cations retained on chelating resins and cation exchange resins. In metal chelate chromatography of proteins sorption occurs by the coordination of surface histidine, cysteine and tryptophan residues to transition metal ions such as Cu^{2+} and Zn^{2+}.

The metal ion must be held in an accessible position away from the matrix so as to ensure efficient adsorption. This was achieved using iminodiacetate groups coupled to epichlorohydrin-activated agarose to form a bis-carboxymethylamino agarose matrix which would then complex with metal cations. The coupling of iminodiacetate to oxirane-activated agarose may be achieved in the laboratory (59,61) (*Method Table 12*). However, iminodiacetate-substituted agarose is also available from Pharmacia-LKB. Iminodiacetate has also been coupled to other supports including Trisacryl GF 2000, a macroporous polyhydroxymethyl polymer (62) and epoxysilylated silica for use in high performance metal chelate chromatography (63). Chelating Sepharose-6B (Pharmacia-LKB) has been used to purify a large number of proteins, including trypsin isoinhibitors (64), mammalian interferons (65), fibronectin (66), macroglobulin (76) and albumin (68).

7.1 Theory of metal chelate chromatography

The complexation of the transition metals zinc and copper with histidine imidazole and cysteine thiol groups (59) is pH-dependent with neutral pH conditions providing the strongest level of adsorption. Under alkaline conditions coordination with amino groups provides a stronger but less selective adsorption. Other metal ions which can coordinate with histidine and cysteine residues include cobalt, nickel, manganese, cadmium, magnesium and calcium, although the affinity of proteins for these cations is generally less than for copper and zinc (69). The complexation of tryptophan residues with these metal cations is also suspected (65).

Metal chelate chromatography therefore differs from conventional purification methods such as ion exchange and sorption since the interaction between protein and matrix does not take place directly but occurs via the complex-forming metal ion (60). The complex once formed must be sufficiently stable so that protein binding can occur irrespective of salt and non-electrolyte concentration. The technique thus separates proteins according to the surface density and number of imidazole and thiol residues and therefore involves a highly selective sorption process.

Method Table 12. Method for the preparation of iminodiacetate-substituted agarose.

1. Add 125 g of epoxy-activated agarose to 100 ml of 2 M sodium carbonate containing 20 g of the disodium salt of iminodiacetic acid.
2. Shake gently for 24 h at 60–65°C.
3. Cool and wash the gel on a glass filter funnel using:
   (i) 0.1 M sodium carbonate;
   (ii) 0.01 M sodium acetate;
   (iii) water.

Method Table 13. Procedure for protein purification using metal chelate chromatography.

1.	Degas matrix and pack into column.
2.	Charge with metal ion in water (e.g. $ZnCl_2$, or $CuSO_4 \cdot 5H_2O$, 1 mg ml^{-1}). The most appropriate metal ion should be determined empirically beforehand.
3.	Equilibrate the column with a neutral pH buffer containing salt to minimize electrostatic effects (e.g. 0.02 M phosphate, pH 7.5 + 0.5 M NaCl).
4.	Apply the sample pre-equilibrated in the same buffer.
5.	Wash the column with equilibration buffer.
6.	Elute protein using a reduced pH buffer e.g. 0.1 M sodium acetate, pH 4–6 + 0.5 M NaCl.
7.	Elute strongly bound proteins using a chelating agent (e.g. 50 mM EDTA in 50 mM sodium phosphate buffer pH 7 + 0.5 M NaCl).

Subsequent elution is commonly achieved using a lower pH which destabilizes the protein–metal chelate complex (68). Although chelating agents (e.g. EDTA) provide little selective elution, they are frequently used to remove more strongly bound proteins (59,64,66). The most appropriate metal ion for a particular application must generally be determined empirically (69). Porath and co-workers (59) found that for serum protein purification copper and zinc ions were most effective and these are the metal ions of choice in most applications.

While much data exists on the binding of metal ions to proteins in free solution it is not always applicable to sorption onto immobilized metal chelates (65). Human serum albumin binds to zinc via almost all of its 16 imidazole groups in free solution, but it is not retained on a zinc chelate matrix.

7.2 Experimental conditions

The procedure for protein adsorption using metal chelate adsorbents is shown in *Method Table 13*.

A similar quantity of agarose gel to that used in ion exchange is recommended (69), while a flow rate of 20–25 ml cm^{-2} h^{-1} should provide optimum resolution (68). A range of metal ions should be tested initially on a protein sample to determine the most suitable conditions. This is best achieved using a series of small test columns each pre-equilibrated with a different metal ion (e.g. $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, $MnCl_2$, $CuSO_4$: each at 1–5 mg ml^{-1}). As mentioned previously, protein adsorption is best achieved under neutral conditions in the absence of chelating agents. If the metal ion–protein binding is strong, the chelating properties of Tris–HCl buffer can be used to reduce the strength of binding. Salt (0.5–1.0 M NaCl) should be added in all buffers to minimize electrostatic interaction (68).

Elution is typically achieved using 0.05–0.1 M sodium acetate buffer at pH 4–6: the lower the pH the more effective the elution.

Displacement elution conditions using imidazole, histidine, glycine or ammonium chloride can also be used, while 0.05–0.1 M Tris–HCl, pH 8 or Tris–acetate pH 3 may also prove successful (67). All elution buffers should contain salt.

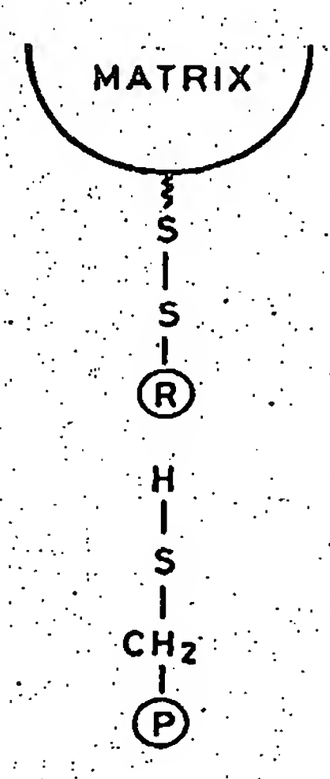


Figure 35. Principle of covalent chron
residue reduces the ligand disulphide wi
is formed between the protein surface

EDTA (0.05 M) should be us
bound protein and regenerate t

8. COVALENT CHROMATO

Covalent chromatography was
isolation of thiol-containing prot
a chemical reaction between the
(71). Covalent chromatography i
proteins by thiol–disulphide int
workers in 1973 (72,73). Protein
by intra- and inter-chain disulp
residues. However, many protei
can be oxidized to form a mixe
tionary phase (*Figure 35*).

The isolation of proteins by t
a matrix-attached disulphide 2′-p

The chromophoric pyridine-2-t
at 343 nm, allowing the adsor
containing proteins are bound,
elution promoted by reducing a
(74). After use the matrix is re-ac
cross-linked dextran and polyacry
chromatography, agarose has bee
of separations (75).

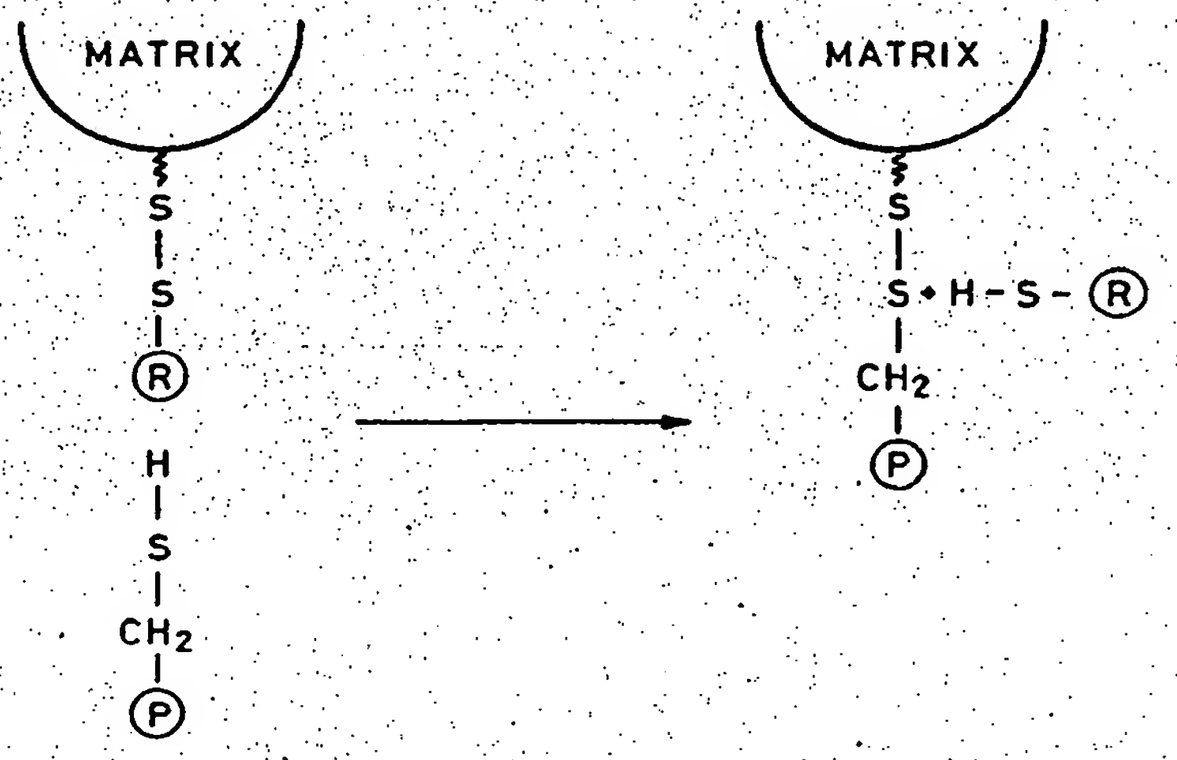


Figure 35. Principle of covalent chromatography. A protein (P) with a surface thiol provided by a cysteine residue reduces the ligand disulphide with the release of the end-capping functionality (R). A new disulphide is formed between the protein surface cysteine and the matrix thiol.

EDTA (0.05 M) should be used at the end of every purification to remove strongly bound protein and regenerate the column by stripping off bound metal ions.

8. COVALENT CHROMATOGRAPHY

Covalent chromatography was originally developed by Eldjam and Jellum (70) for the isolation of thiol-containing proteins using organomercurial-polysaccharide and involves a chemical reaction between the chromatographic material and a component in solution (71). Covalent chromatography is now generally used for the isolation of thiol-containing proteins by thiol–disulphide interaction, a method introduced by Bocklehurst and co-workers in 1973 (72,73). Protein tertiary and quaternary structure is frequently stabilized by intra- and inter-chain disulphide bridges formed by the oxidation of two cysteine residues. However, many proteins have non-oxidized surface cysteine side chains which can be oxidized to form a mixed disulphide with a secondary thiol attached to a stationary phase (*Figure 35*).

The isolation of proteins by thiol–disulphide interchange involves the reaction of a matrix-attached disulphide 2′-pyridyl group with a thiol-containing protein (*Figure 36*).

The chromophoric pyridine-2-thione released can be monitored spectrophotometrically at 343 nm, allowing the adsorption of proteins to be simply assessed. After thiol-containing proteins are bound, loosely adsorbed contaminants are washed away and elution promoted by reducing agents such as cysteine, glutathione and dithiothreitol (74). After use the matrix is re-activated with 2,2′-dipyridyl disulphide. While cellulose, cross-linked dextran and polyacrylamide have all been used as matrix supports in covalent chromatography, agarose has been adopted as the matrix of choice in the largest number of separations (75).

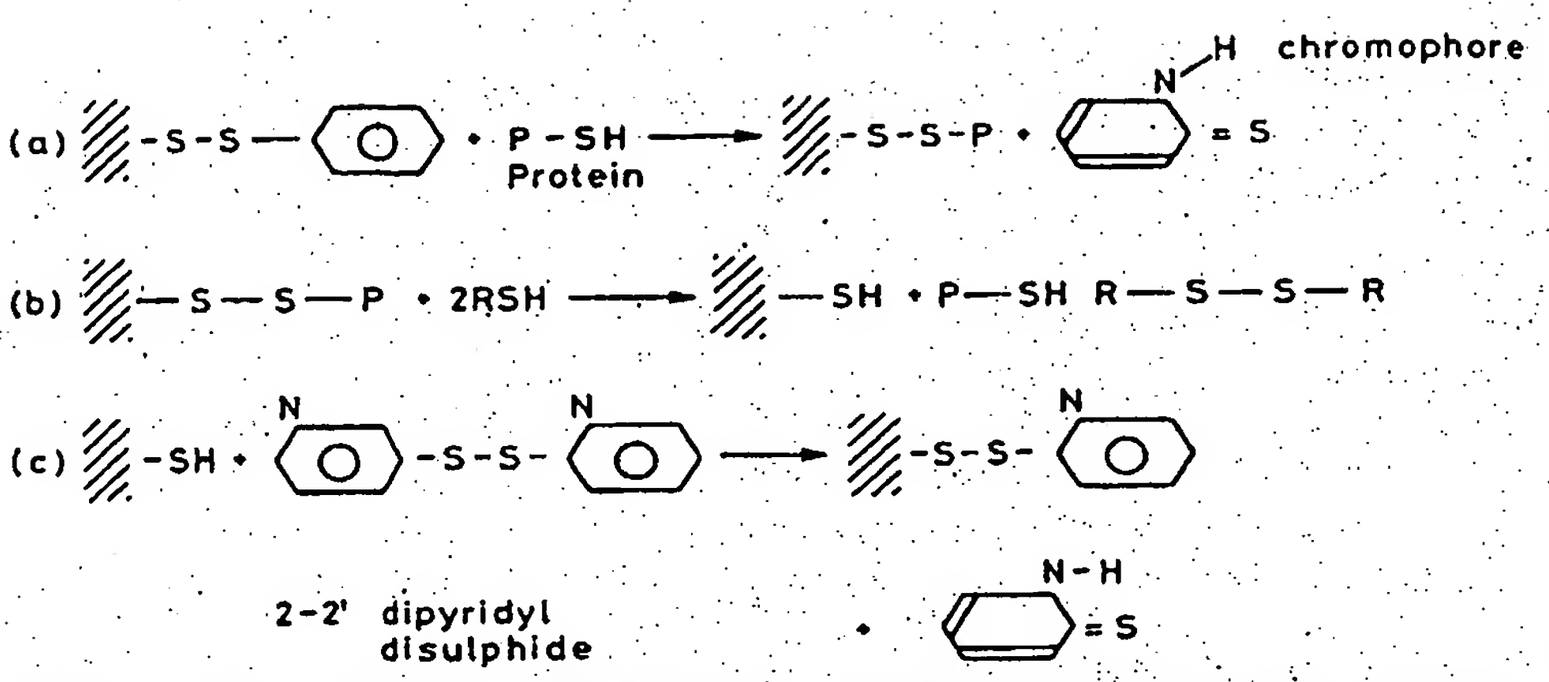


Figure 36. Adsorption, elution and regeneration steps in covalent chromatography by thiol–disulphide interchange. Reproduced with kind permission of Pharmacia-LKB.

8.1 Experimental techniques

8.1.1 *Adsorption*

This separation technique is usually applied to partially purified products with minimal non-protein contamination. It is therefore used following initial sample clean-up using salt-precipitation and ion-exchange chromatography (74). There are cases, however, where it has been used for protein isolation from relatively crude preparations (76).

An example of a procedure for covalent chromatography using Thiopropyl Sepharose is shown in *Method Table 14*. The pyridyl disulphide is attached to the cross-linked agarose matrix by a hydroxypropyl spacer arm. For the separation of larger proteins, activated Thiol Sepharose-4B is preferred. The lower degree of cross-linking and the longer spacer arm used in Thiol Sepharose (glutathione) allow adsorption of larger proteins. The gel capacity is, however, rather lower than for Thiopropyl Sepharose-6B. Adsorption can be carried out using column or batch techniques. Both initial gel pre-treatment and sample application use mild conditions with buffering at neutral or mildly acidic pH (74). At pH 8 most thiol-containing proteins will react readily while at an acidic pH (~4) the selectivity of protein binding can be increased by the adsorption of proteins having an abnormally low pK_a value. This includes many cysteine proteinases such as papain which was selectively adsorbed at a low pH by Brocklehurst and co-workers (73). Papain has an active site cysteine residue which has an unusually low pK_a value due to intramolecular hydrogen bonding.

8.1.2 *Elution*

Elution of bound proteins is achieved using low molecular weight thiols at a neutral pH. Removal of unreacted thiopyridyl groups prior to protein elution can, however, be accomplished using 4 mM dithiothreitol or 2-mercaptoethanol in 0.1 M sodium acetate, pH 4 (74). Protein elution generally uses 20–50 mM dithiothreitol or 2-mercaptoethanol in a neutral pH buffer.

Method Table 14. Procedure for cc
Sepharose-6B.

1.	Swell gel for 15 min at room sodium phosphate, pH 7).
2.	Pack into column and wash
3.	Apply sample and wash off c step can be monitored at 34
4.	Elute bound protein sequent
	(i) 5–25 mM L-cysteine 7–8.
	(ii) 50 mM reduced gluta
	(iii) 20–50 mM 2-mercap
	(iv) 20–50 mM dithiothr

Hillson (77) developed a sequen during the desorption step. By usin or increasing concentrations of the sa proteins can be improved (*Method*

8.1.3 *Regeneration and storage*

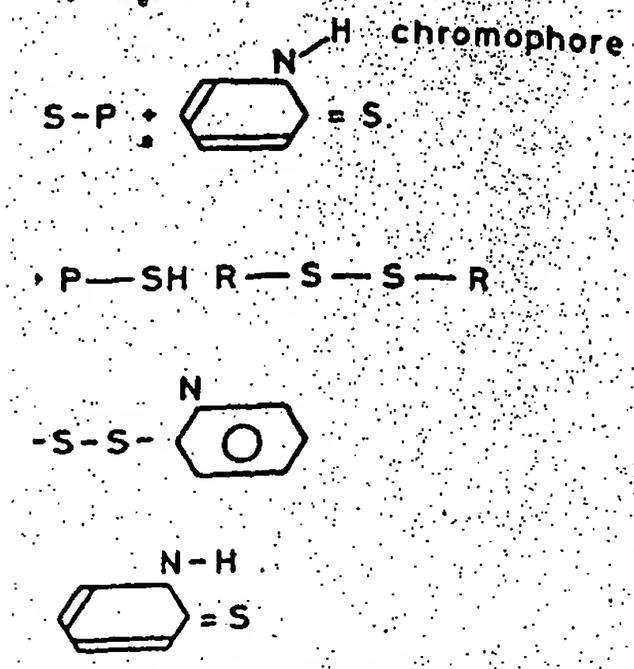
Following protein elution, reducin 30–40 mg ml^{-1} 2,2′-dipyridyl di recommended procedure for Thiop

(i) Mix one volume of 2,2′-dip isopropanol with four volum EDTA.
(ii) Reflux at 80°C for 3 h.
(iii) Wash gel in ethanol and re-

After use agarose gels should be sto 0.02% sodium azide. Do not all preservatives should be avoided.

8.2 Advantages and disadvantage

Covalent chromatography is a use techniques currently available. Its sel been used in a large number of app further increased by adjustment of th method. The high degree of specific in relatively crude applications (74 chromatography is limited by its co of gel matrix, the lengthy regenerat drawback in its use.



lent chromatography by thiol–disulphide
KB.

lly purified products with minimal
wing initial sample clean-up using
' (74). There are cases, however,
elatively crude preparations (76).
raphy using Thiopropyl Sepharose
ide is attached to the cross-linked
the separation of larger proteins,
er degree of cross-linking and the
hione) allow adsorption of larger
than for Thiopropyl Sepharose-6B.
h techniques. Both initial gel pre-
with buffering at neutral or mildly
eins will react readily while at an
an be increased by the adsorption
ıe. This includes many cysteine
orbed at a low pH by Brocklehurst
ine residue which has an unusually
ıding.

olecular weight thiols at a neutral
r to protein elution can, however,
nercaptoethanol in 0.1 M sodium
ses 20–50 mM dithiothreitol or

Method Table 14. Procedure for covalent chromatography of proteins using Thiopropyl Sepharose-6B.

1. Swell gel for 15 min at room temperature in a buffer at neutral pH (e.g. 20 mM sodium phosphate, pH 7).
2. Pack into column and wash through with the same buffer.
3. Apply sample and wash off contaminants with the starter buffer. The adsorption step can be monitored at 343 nm.
4. Elute bound protein sequentially with:
   (i) 5–25 mM L-cysteine + 1 mM EDTA in a neutral buffer, pH 7–8.
   (ii) 50 mM reduced glutathione + 1 mM EDTA in elution buffer.
   (iii) 20–50 mM 2-mercaptoethanol + 1 mM EDTA in elution buffer.
   (iv) 20–50 mM dithiothreitol + 1 mM EDTA in elution buffer.

Hillson (77) developed a sequential elution method allowing increased selectivity during the desorption step. By using a series of thiols of increasing reducing strength or increasing concentrations of the same thiol, the degree of resolution of thiol-containing proteins can be improved (*Method Table 14*).

8.1.3 *Regeneration and storage*

Following protein elution, reducing agents must be displaced from the gel using 30–40 mg ml^{-1} 2,2′-dipyridyl disulphide pH 8 (activated Thiol Sepharose). The recommended procedure for Thiopropyl Sepharose is as follows.

(i) Mix one volume of 2,2′-dipyridyl disulphide (30–40 mg ml^{-1}) in ethanol or isopropanol with four volumes of gel in 0.1 M borate buffer pH 8 + 1 mM EDTA.
(ii) Reflux at 80°C for 3 h.
(iii) Wash gel in ethanol and re-equilibrate in starting buffer.

After use agarose gels should be stored refrigerated at pH 4 in a sealed bottle containing 0.02% sodium azide. Do not allow the pH to drop below 4. Thiol-containing preservatives should be avoided.

8.2 Advantages and disadvantages

Covalent chromatography is a useful addition to the range of protein separation techniques currently available. Its selectivity for removing thiol-containing proteins has been used in a large number of applications. The specificity of the technique can be further increased by adjustment of the adsorption pH and by using the sequential elution method. The high degree of specificity during the adsorption stage may allow its use in relatively crude applications (74) but at present the large-scale use of covalent chromatography is limited by its cost (71). Although this is offset by the re-usability of gel matrix, the lengthy regeneration procedure involving a 3 h reflux step, is one drawback in its use.

9. OTHER ADSORPTION TECHNIQUES

Apart from the adsorption phenomena already described, other mediators of protein adsorption include the following.

(i) Hydrogen bonds formed by the interaction of a hydrogen atom covalently bonded to an electronegative atom with another electronegative atom having a lone electron pair.
(ii) Van der Waals forces caused by the perturbations in the electron clouds of molecules.
(iii) Dipole interactions between molecules with partial charge separations.

Many different types of adsorbents thought to interact via these forces have been used in protein purification, for example, titania, alumina, Fullers Earth, Bentonite and Celite. However, since the precise nature of the adsorption mechanism is not clear an empirical approach is frequently used in the choice of adsorption and elution conditions. Most of these materials have been used in the form of particulates and have the advantage of being relatively inert, easily sterilized, cheap and of low toxicity (8). However, significant problems are associated with their use including irreversible adsorption, column clogging, variable capacities and slow adsorption/desorption kinetics. With the advent of superior matrices based on polysaccharides such as cellulose their use is therefore limited. Only one adsorbent, hydroxylapatite, continues to attract much attention and its use is described below.

9.1 Hydroxylapatite

Calcium phosphate gels have been used in protein purification for some time (8). However, it was not until the development of crystalline calcium phosphate or hydroxylapatite by Tiselius and co-workers (78,79) that the flow characteristics of this adsorbent were improved sufficiently to allow its successful use in column chromatography. Nevertheless, the widespread use of hydroxylapatite (HA) has in the past been limited, due in part to predictability, the availability of alternative adsorbents with superior chromatographic properties and the lengthy laboratory preparation necessary for its use (80).

Crystalline hydroxylapatite [$Ca_{10}(PO_4)_6(OH)_2$] is prepared by slowly mixing calcium chloride and sodium phosphate to form a precipitate of brushite ($Ca_2\ HPO_4 \cdot 2H_2O$). The brushite is then boiled with sodium hydroxide or ammonia to convert it to hydroxylapatite. The mechanism of protein adsorption onto HA is thought to involve both Ca^{2+} and PO_4^{3-} groups on the crystal surface (81,82). Since these charged groups are closely arranged on the crystal it is likely that dipole–dipole interactions exist between adsorbent and protein although purely electrostatic interactions cannot be ruled out. Bernadi (80,82) has suggested that acidic and neutral proteins bind to the hydroxylapatite calcium while basic proteins adsorb to surface phosphate groups. Exceptions to this rule include the phosphate binding capability of certain acidic proteins such as glycolytic and pentose phosphate pathway enzymes and aminoacyl tRNA synthetases.

Commercially available hydroxylapatites (83,84,85) and calcium phosphate-coated gels are now available from several manufacturers (*Table 20*), eliminating the need for laboratory preparation of HA. It should be noted, however, that commercial HA may be inferior to fresh laboratory-prepared material.

Table 20. Characteristics of some commercially available hydroxylapatite preparations.

Commercial name	*Manufacturer*	*Matrix*	*Typical capacity (mg ml^{-1})*	*Additional data*
HA-Ultrogel	IBF	Agarose	10 (Cyt *c*) 2 (BSA)	Bead diameter 60–180 μm Exclusion limited 5 × 10^6 (globular

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HA-Ultrogel	IBF	Agarose	10 (Cyt *c*) 2 (BSA)	Bead diameter 60–180 μm Exclusion limited 5 × 10^6 (globular proteins)
Biogel HT/HTP	Bio-Rad	Crystalline HA	10 (BSA)	Prepared by the conventional method
Macrosorb C	Sterling Organics	Fabricated Macroporous HA	7 (BSA)	Pore size 1000 nm Bead diameter 250–500 μm
Hydroxylapatite-spheroidal	BDH	Spheroidal HA	2.5 (BSA)	Has greater mechanical stability than crystalline HA
Bio-Gel HPHT	Bio-Rad	Crystalline HA	5 mg ml^{-1} for optimum resolution	High performance material

Separation based on structure

Since hydroxylapatite adsorbs proteins by a different mechanism to other separation techniques, it is a useful additional chromatographic procedure which can often be used to provide resolution of protein mixtures not achievable by alternative methods such as ion exchange and hydrophobic interaction chromatography.

9.1.1 *Adsorption conditions*

The adsorption of proteins using HA can be carried out using batch or column methods. For large volume samples the former should be preferred. Adsorption is typically carried out in a low concentration of sodium or phosphate buffer (<20 mM) at a neutral pH (80,86). For batch conditions an adsorption time of 30 min has been used with gentle agitation. It is essential to avoid the presence of substances with a stronger affinity for calcium than phosphate (eg. EDTA and citrate), since this will decrease the hydroxylapatite capacity (80). In column chromatography the hydroxylapatite may be pre-washed in 0.5 M phosphate buffer pH 6.8 to ensure the removal of any adsorbed contaminants (86). The HA is then washed in a low concentration of phosphate buffer (pH 6.5–7) prior to sample application. While the adsorption of acidic proteins is little affected by the presence of sodium, potassium or calcium chloride, these salts may reduce the capacity of HA for basic proteins (80). Lysozyme, a basic protein, will not adsorb onto HA in the presence of 2 M KCl (80).

Typical flow rates used in column chromatography are 10–25 ml cm^{-2} h^{-1} with crystalline hydroxylapatite but the BDH spherical porous material may be used at up to 100 ml cm^{-2} h^{-1}.

The capacity of HA for proteins is generally at its highest close to neutrality. Atkinson *et al.* (86), showed that the capacity of laboratory-prepared material for bovine plasma albumin was relatively constant at about 40 mg ml^{-1} of HA between pH 6.5 and 7, but fell off rapidly above pH 7 (86). Commercially available hydroxylapatite preparations have quoted capacities of nearer 10 mg ml^{-1} for protein (84,85). Following adsorption, the hydroxylapatite is usually washed with 1–2 volumes of a low concentration of phosphate buffer (~20 mM).

9.1.2 *Elution conditions*

Protein elution from hydroxylapatite is usually achieved with a stepwise or continuous gradient of increasing phosphate concentration up to 500 mM (80,86). Potassium phosphate is preferred to sodium phosphate at higher concentration and at 4°C due to its superior solubility.

Basic proteins (e.g. lysozyme) absorb strongly to HA and are usually eluted at a phosphate concentration above 120 mM, pH 6.8. Acidic and neutral proteins may be eluted at lower concentrations (30–120 mM). Very basic proteins (e.g. lysine-rich histones) may require much stronger eluting conditions of up to 500 mM phosphate. An example of a procedure (87) for the fractionation of proteins using HA is given in *Method Table 15*. The stepwise elution of proteins using an increasing concentration of phosphate may lead to severe peak tailing when used in column chromatography (80) and is best used under batch conditions.

The pH of the eluent may have a significant effect on the concentration of phosphate required to desorb a protein. Atkinson and coworkers (86), found that although

Method Table 15. Procedure for

1.	Suspend the hydroxylapatite of dry material should be u
2.	Pour the hydroxylapatite slur and wash the column throu phosphate, pH 6.8, using a
3.	Apply the protein sample, p
4.	Wash the bed with two bed
5.	Elute the protein with a linea e.g. 0.02–0.5 M potassium normally be used.
6.	Remove tightly bound protein

NB Purifications carried out in the in concentration.

Column :	Bi
Flow rate :	1 r
Gradient :	0. 0. to
Sample :	0.

Figure 37. HPLC purification of monoclonal from Bio-Rad.

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buffer (<20 mM) at a neutral pH
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Method Table 15. Procedure for the fractionation of proteins using hydroxylapatite.

1. Suspend the hydroxylapatite in 20 mM potassium phosphate buffer pH 6.8. 1 g of dry material should be used per 10 mg of protein.
2. Pour the hydroxylapatite slurry into the column (typical dimensions 2.5 × 25 cm) and wash the column through with five bed volumes of 20 mM potassium phosphate, pH 6.8, using a flow rate of ~20 ml cm^{-2} h^{-1}.
3. Apply the protein sample, preferably in the starting buffer.
4. Wash the bed with two bed volumes of starting buffer at 20 ml cm^{-2} h^{-1}.
5. Elute the protein with a linear gradient of an increasing phosphate concentration, e.g. 0.02–0.5 M potassium phosphate, pH 6.8. Up to 10 bed volumes should normally be used.
6. Remove tightly bound protein by continuing to elute with 0.5 M buffer at pH 6.8.

NB Purifications carried out in the cold should not use phosphate buffers over 0.5 M in concentration.

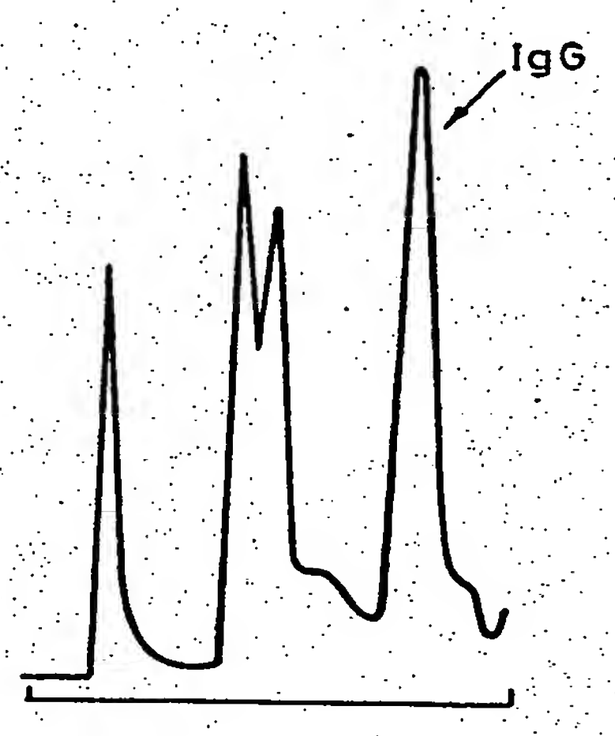
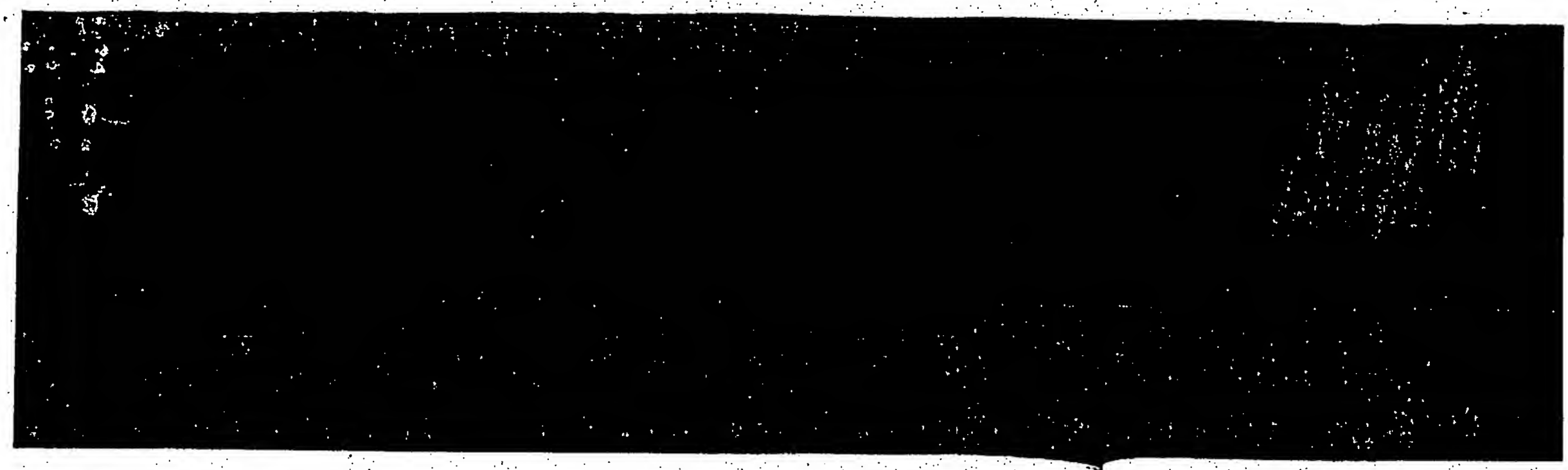


Figure 37. HPLC purification of monoclonal antibodies using hydroxylapatite. Reproduced with permission from Bio-Rad.



tryptophan–tRNA synthetase, an acidic protein, requires a surprisingly high phosphate concentration (400 mM) to promote elution at pH 6.8, a concentration of less than 250 mM is adequate at pH 7.5. Optimum resolution is obtained using hydroxylapatite in columns with a length: diameter ratio of between 5:1 and 15:1. Hydroxylapatite has a tendency to adsorb carbon dioxide to form a hard crust as a layer on the column top. This may cause an increase in back pressure and reduce the flow rate. The top layer (~1 cm) of the column should therefore occasionally be removed. Bio-Rad also recommend that all water used in buffers should be boiled to remove carbon dioxide.

9.1.3 *Regeneration and storage*

Following elution the column should be washed thoroughly with 0.5 M phosphate buffer, pH 6.8 and then re-equilibrated in the starting buffer. Hydroxylapatite should be stored in a low concentration of phosphate buffer containing 0.03% (v/v) toluene or 0.03% (w/v) sodium azide.

9.2 High performance hydroxylapatite

Bio-Rad also market a hydroxylapatite material pre-packed in a stainless steel column for use in HPLC. The analytical scale column (4.8 ml bed volume) will purify 20 mg of protein per separation in less than 2 h using a flow rate of 0.5–1 ml min^{-1} (83). Purification of larger amounts of protein (up to 100 mg per injection) necessitates the use of preparative-scale HPLC (5 × 2.5 cm column). A chromatogram for the purification of monoclonal antibodies using high performance hydroxylapatite is shown in *Figure 37*. The conditions for protein separation, using an increasing concentration of phosphate for elution, are similar to those used in low pressure chromatography.

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Purification by

1. INTRODUCTION—S.Angal an

Proteins carry out their biological fu
consequently contain binding sites fo
Ligands may be small molecules su
such as peptide hormones. The inter
by the overall size and shape of the
complementary surfaces. These co
of charged and hydrophobic moieties
such as hydrogen bonds. This bind
and often of a high affinity, can be
technique commonly known as aff

The operation of affinity chroma

(i) Choice of an appropriate lig
(ii) Immobilization of the ligand
(iii) Contacting the protein mixtu
(iv) Removal of non-specifically
(v) Elution of the protein of int

At best, affinity chromatography is t
since its high selectivity can, in prin
abundance from a crude mixture of
affinity of the ligand for the protein i
concentration from a large volume.
common and successful affinity ch
number of parameters involved. Th
experimenter in the selection and
For more extensive information on
many excellent texts on this subject

2. DESIGN AND PREPARATIO

P.D.G.Dean

The construction of an affinity ads
involves three major factors.

(i) Choice of a suitable ligand.
(ii) Selection of a support matri
(iii) Attachment of the ligand to

The criteria for making these deci